UNIVERSITY OF ALBERTA

SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL 1,4-DIHYDROPYRIDINE – NITRIC OXIDE DONOR HYBRIDS

BY

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This work is dedicated to

My father, Nguyen Huu-Te,
My mother, Tran Van Thi My-Linh,
My brother, Nguyen Huu-Tam,
My sister, Nguyen My-Lien,
My brother-in-law, Van Thanh Phu,
My nephews, Van Thanh Khang
Van Thanh Duy.

ABSTRACT

A group of racemic 3-(nitrooxyalkyl or 4-chlorobutyl) 5-alkyl 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**6-17**), 5-[(3-benzenesulfonyl)furoxan-4-yloxy]alkyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl, benzofurazan-4-yl, 2-, 3- or 4-pyridinyl)-5-pyridinecarboxylates (**24-38**), 3-isopropyl 5-(2-piperazinylethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**43-45**), 3-isopropyl 5-(2-[4-nitrosopiperazinyl]ethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**46-48**) and 3-isopropyl 5-(2-[(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)(*N*,*N*-dialkylamino or 4-piperazin-1-yl)]ethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**57-65**) was prepared using modified Hantzsch reactions.

All the 1,4-dihydropyridine calcium channel antagonists (**6-17**, **24-38**, **43-44**, **46-48**, and **57-65**) exhibited less potent calcium channel antagonist activity (IC_{50} = 0.03 μM to > 29.91 μM) than the reference drug, nifedipine (IC_{50} = 0.01 μM). The C-3 alkyl was a primary determinant of calcium channel antagonism (CO_2*i*-Pr >> CO_2*i*-Bu > CO_2*t*-Bu > CO_2Et > NO_2). The point of attachment of the isomeric C-4 substituent was a determinant of calcium channel antagonist activity providing the potency profile 2-pyridinyl > 3-pyridinyl and 4-pyridinyl > 4-benzofurazanyl > 2-trifluoromethylphenyl. Calcium channel antagonism with respect to the C-5 substituent was 2-nitrooxyethyl > 2-[*N*-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-methylamino]ethyl and 1,3-dinitrooxy-2-propyl > 3-nitrooxypropyl, 4-chlorobutyl and 2-piperazinylethyl > 4-nitrooxybutyl and 2-[4-nitrosopiperazinyl]ethyl > 2-[*N*-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-(*n*-butyl)amino]ethyl > 4-[3-(benzenesulfonyl)furoxan-4-yloxy]butyl and 3-[3-(benzenesulfonyl)furoxan-4-yloxy]propyl > 2-[3-(benzenesulfonyl)furoxan-4-yloxy]ethyl,

2-[*N*-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl and 2-[4-nitrosopiperazinyl]ethyl.

None of the 1,4-dihydropyridine calcium channel agonists (**24-38**) exhibited calcium channel agonist activities (EC_{50} = 3.02 to > 44.66 μM) that were greater than the reference drug, BAY K 8644 (EC_{50} = 0.77 μM). Compounds **27** and **29-33** were more potent than the reference drug, PN 202 791 (EC_{50} = 9.40 μM). The order of calcium channel agonist potency for compounds **24-38**, with respect to substituents at the C-4 position, was 4-benzofurazanyl > 3-pyridinyl > 4-pyridinyl > 2-pyridinyl > 2-trifluoromethylphenyl. Substitution at the C-5 position did not greatly affect agonist activity.

The extent of nitric oxide released from compounds **6-16**, **24-38** and **57-65** ranged from 33 times less to 7.2 times more than the reference drug, glyceryl trinitrate, (11% mol/mol using *N*-acetylcysteamine; 20% mol/mol using L-cysteine). The extent of nitric oxide released *in vitro* was dependent upon the specific nitric oxide donor moiety (N^1-[4-piperazinyl, ethyl, *n*-butyl] diazen-1-ium-1,2-diolate > [3-benzenesulfonyl]furoxan-4-yl > N^1-methyl diazen-1-ium-1,2-diolate > nitrooxy). *N*-Nitrosoamine hybrids (**46-48**) did not release nitric oxide.

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LIST OF ABBREVIATIONS

δ	In an NMR context, "δ" denotes "chemical shift" expressed in parts-per-million (ppm).
AM1	A semi-empirical molecular orbital conformational analysis
Anal. Calcd.	Microanalytical calculations
C-2	Position C-2 of the 1,4-dihydropyridine ring
C-3	Position C-3 of the 1,4-dihydropyridine ring
C-4	Position C-4 of the 1,4-dihydropyridine ring
C-5	Position C-5 of the 1,4-dihydropyridine ring
C-6	Position C-6 of the 1,4-dihydropyridine ring
$CDCl_3$	Deuterated chloroform
^{13}C NMR	Carbon-13 nuclear magnetic resonance spectroscopy
conc. H_2SO_4	Concentrated sulfuric acid
cyclic AMP	Cyclic adenosine monophosphate
cyclic GMP	Cyclic guanosine monophosphate
d	In a chemical synthesis context, "d" denotes "days".
d	In a spin multiplet context, "d" denotes "doublet".
dd	In a spin multiplet context, "dd" denotes "doublet of doublets".
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DMAP	4-Dimethylaminopyridine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sufoxide
D_2O	Deuterated water
EC_{50}	50% Effective Concentration. The concentration of a compound that is required to produce 50% of the maximum effect.
Et	Ethyl
ether	Diethyl ether
Et_3N	Triethylamine
EtOAc	Ethyl acetate
EtOH	Ethanol
FMN/NADH	Flavin mononucleotide / reduced β-nicotinamide-adenine dinucleotide

h	Hours
HbO_2	Oxyhemoglobin
HMPA	Hexamethylphosphoramide
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
i-Bu	Isobutyl
IC_{50}	50% Inhibitory Concentration. The concentration of a compound that is required to produce 50% inhibition.
i-PrOH	Isopropanol
IR	Infrared spectroscopy
J or J_{vic}	Vicinal coupling constant (expressed in Hertz)
J_{gem}	Geminal coupling constant (expressed in Hertz)
lit. mp	Literature melting point
m	In a spin multiplet context, "m" denotes "multiplet".
Me	Methyl
MeCN	Acetonitrile
MeOH	Methanol
MetHb	Methemoglobin
min	Minutes
mp	Melting point (°C)
MsCl	Methanesulfonyl chloride
m/z (ES+)	Positive polarity electrospray mass spectroscopy
n	In a statistical context, "n" denotes "the number of test samples".
NADPH	The reduced form of β-nicotinamide-adenine dinucleotide phosphate
NaOMe	Sodium methoxide
n-Bu	Normal-butyl
N-1	Position N-1 of the 1,4-dihydropyridine ring
NMR	Nuclear magnetic resonance spectroscopy
nOe	^{1}H NMR nuclear Overhauser enhancement
OAc	Acetoxy
p	P-value. Probability of error. Statistical significance.
PDE5	Cyclic GMP specific phosphodiesterase type 5
Ph	Phenyl
PM3	A semi-empirical molecular orbital conformational analysis

pS	picoSiemens. Permeability-surface area product. Commonly calculated as current divided by voltage.
psi	Pound per square inch
PTLC	Preparative thin layer chromatography
q	In a spin multiplet context, "q" denotes "quartet".
R^2	In a statistical context, "R^2" denotes "the coefficient of determination".
s	In a spin multiplet context, "s" denotes "singlet".
SEM	Standard error of the mean
S_N2	Nucleophilic substitution type 2
t	In a spin multiplet context, "t" denotes "triplet".
t-Bu	Tertiary-butyl
THF	Tetrahydrofuran
TLC	Thin layer chromatography
(v/v)	Volume per volume mixture
(w/w)	Weight per weight mixture

1 INTRODUCTION

1.1 HEART FAILURE

1.1.1 Epidemiology

In 2001, the Heart and Stroke Foundation of Canada reported that 200 000 to 300 000 individuals in Canada have been diagnosed with heart failure, and as much as 40% of those patients died by the end of the year [Giannetti, 2001]. Heart failure patients have a 5-year survival rate of 62% [Weil & Tu, 2001], and death is classified as sudden in 20% to 50% of the patients [CONSENSUS, 1987]. Heart failure is one of the most common causes of death and disability in industrialized nations [Ooi & Colucci, 2001]. Heart failure is a leading reason for hospitalization admission among the elderly population [Weil & Vu, 2001]. In view of the increase in the geriatric population, heart failure is a medical problem that has become even more significant with time [Johnson *et al.*, 1999]. Indeed, since 1970 until 2001, the rate of death from heart failure in Canada has increased by 60% [Weil & Tu, 2001].

1.1.2 Etiology

Heart failure is a pathophysiological state in which the heart is unable to pump blood at a rate sufficient to meet the metabolic needs of the body [Betkowski & Hauptman, 2000].

Heart failure can be caused by a systolic dysfunction. Such dysfunction is the consequence of reduced myocardial contractility, resulting in lower cardiac output that leads to fatigue and decreased exercise capacity [Ho *et al.*, 1993; Kannel, 1996; CONSENSUS, 1987; SOLVD, 1991; O'Connol & Bristow, 1994; Colucci *et al.*, 1997].

Alternatively, heart failure can be the result of a diastolic dysfunction. Restrictive ventricular filling, which leads to intravascular volume expansion and elevated ventricular filling pressure, causes the dysfunction. These effects cause systemic and pulmonary venous hypertension, which subsequently cause breathing difficulties such as dyspnea[1] on exertion and orthopnea[2] [Ho *et al.*, 1993; Kannel, 1996; CONSENSUS, 1987; SOLVD, 1991; O'Connol & Bristow, 1994; Colucci *et al.*, 1997].

---

[1] Difficult or labored respiration

[2] The plight of a person who can only breathe easily when sitting straight or standing erect

The primary goal of therapy for heart failure is symptomatic relief, because heart failure patients often present various symptoms. Therapy usually consists of improving cardiac output and reducing ventricular filling pressure to alleviate these symptoms. Drug therapy often consists of diuretics, positive inotropic agents (β-adrenergic receptor agonists, digitalis or phosphodiesterase inhibitors) and vasodilators (angiotensin II converting enzyme inhibitors, nitroprusside or glyceryl trinitrate) [Ooi & Colucci, 2001].

1.2 PHARMACOLOGY

1.2.1 Calcium Channel Agonists

1,4-Dihydropyridine calcium channel agonists may be useful in treating heart failure. This class of positive inotropes increases cardiac contractile force without increasing heart rate. β-Adrenergic receptor agonists, digitalis and phosphodiesterase inhibitors all act by increasing cyclic AMP. An increase in cyclic AMP release leads to an increase in heart rate and myocardial oxygen consumption. A high cyclic AMP release also worsens the risk of arrhythmia. Although these inotropes can improve hemodynamics and symptoms for short-term therapies, they may not show long-term benefits in improving patient survival [Holland *et al.*, 1989]. β-Adrenergic receptor agonists can induce tachyphylaxis[3] resulting from β-receptor down regulation, and they can also have negative inotropic effects [Robertson *et al.*, 1985]. Digitalis cardiac glycosides have a low therapeutic index of 2 to 3, erratic absorption, harmful drug-drug interactions and often limited efficacy [DIG, 1997]. Phosphodiesterase inhibitors such as amirinone and milrinone can cause thrombocytopenia[4] and an increased incidence of anginal attacks [Baim *et al.*, 1983].

Calcium channel modulators, which do not act via a cyclic AMP mechanism, alter calcium ion concentration that regulates muscular contraction [Triggle, 1992; Natale *et al.*, 1999; Kevins & Robertson, 2001].

However, calcium channel agonists have not undergone clinical trial for the treatment of heart failure, because they are not sufficiently cardioselective [Holland *et al.*, 1989; Gross *et al.*, 1990]. In experimental studies with calf cardiac Purkinje fibers, the calcium channel agonist BAY K 8644 (Figure 1.4.1.3b) showed agonistic properties

---

[3] A rapidly decreasing response to a drug following administration of the initial doses

[4] A blood disease characterized by an abnormally small number of platelets in the blood

at membrane-holding potentials less than -50 mV, but acted as an antagonist at less than -45 mV [Sanguinetti & Kass, 1984a]. Moreover, at high concentration, the calcium channel agonist behaved as a calcium channel antagonist [Holland *et al.*, 1989; Triggle & Rampe, 1989; Gross *et al.*, 1990]. Therefore, although calcium channel agonists improve cardiac inotropy, their adverse vasocontrictive action makes them unsuitable for the treatment of heart failure [Erhardt, 1987; Goldenberg & Cohn, 1987; Holland *et al.*, 1989] (Table 1.2.1).

Table 1.2.1: The Properties of Calcium Channel Modulators

	Agonist Property	**Antagonist Property**
Cardiac Inotropy[5]	Positive (stronger)	Negative (weaker)
Cardiac Chronotropy	No effect	Reflex tachycardia[6]
Cardiac Dromotropy[7]	Unknown (possible prolongation)	No effect
Blood Vessels	Vasoconstriction	Vasodilation

1.2.2 Calcium Channel Antagonists

Calcium channel antagonists may be useful in treating heart failure [CPhA, 2003] (Table 1.2.2). Calcium channel antagonists induce arterial vasodilation thereby decreasing blood pressure, and reducing cardiac afterload [Kevins & Robertson, 2001]. In simple terms, afterload is the resistance of active forward ejection of blood by the ventricle. However, in clinical practice, calcium channel antagonists fail to meet expectations.

The first generation 1,4-dihydropyridine calcium channel antagonist, nifedipine (Table 1.2.2), does not produce a sustained improvement in symptoms in patients with predominant systolic ventricular dysfunction. Because nifedipine has a negative inotropic effect and induces reflex neurohumoral activation [Ooi & Colucci, 2001], nifedipine may even worsen morbidity and increase the mortality rate in patients with

---

[5] The force exerted during a cardiac contraction

[6] An increase in heart rate

[7] The velocity of conduction of each heart beat affecting the PQRST intervals (Figure 1.2.2)

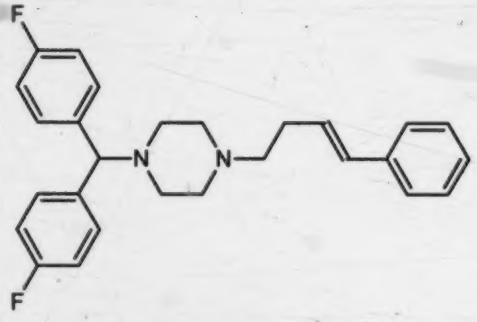
systolic dysfunction caused by ischemic diseases [Elkayam *et al.*, 1990; Elkayam *et al.*, 1993].

The second generation 1,4-dihydropyridine calcium channel antagonists, amlodipine and felodipine (Table 1.2.2), posseses a lower negative inotropic effect, greater cardiovascular selectivity, no sympathetic nervous system activation, and minimal reflex tachycardia [Johnson *et al.*, 1999; Ooi & Colucci, 2001].

Table 1.2.2: Calcium Channel Antagonists Available in Canada.

Non-Antihypertensive Calcium Channel Antagonists

Flunarizine



Therapeutic Indication

- Prophylaxis for migraine with or without aura
- Not for acute migraine attack

Canadian Trade Name (Manufacturer)

- **Dosage Form**

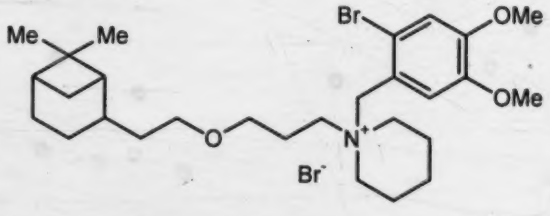
Sibelium (Pharmascience)

- oral tablet

Pharmacology

- Prevents deleterious effects of cellular calcium ion overload by decreasing excessive transmembrane fluxes of calcium ions
- Does not interfere with normal cellular calcium ion
- Antihistaminic properties

Pinaverium Bromide



Therapeutic Indication

- Irritable bowel symptoms: abdominal pain, bowel disturbances and intestinal discomfort
- Functional disorder of the biliary tract

Canadian Trade Name (Manufacturer)

- **Dosage Form**

Dicetel (Solvay Pharma)

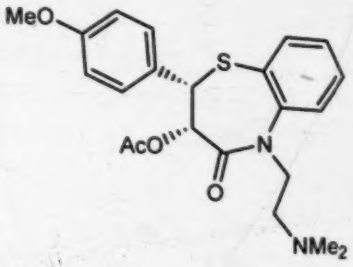
- oral tablet

Pharmacology

- Inhibits calcium influx by blocking the voltage-dependent L-type calcium channel
- High degree of selectivity for intestinal smooth muscle

Anti-Hypertensive Calcium Channel Antagonists

Diltiazem



Canadian Trade Name (Manufacturer)

- **Dosage Form**

Cardizem (Biovail Pharmaceuticals)

- Regular-release, controlled-release or sustained-release oral tablet

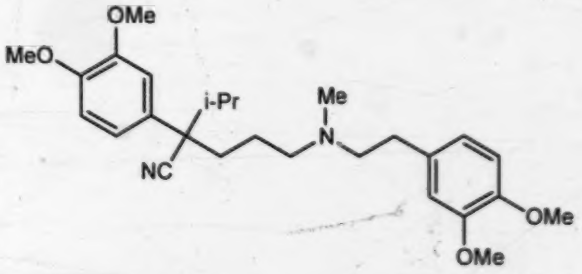
Therapeutic Indication

- Angina resulting from coronary spasm
- Chronic stable angina (effort associated angina)
- Mild to moderate essential hypertension

Pharmacology

- Inhibits calcium influx by blocking the voltage-dependent L-type calcium channel

Verapamil



Canadian Trade Name (Manufacturer)

- **Dosage Form**

Isoptin (Abbott)

- Intravenous injection
- Regular-release or sustained-release oral tablet

Tarka (Abbott)

- Oral tablet containing verapamil and the angiotensin II converting enzyme inhibitor trandolapril

Therapeutic Indication

- Angina resulting from coronary spasm
- Chronic stable angina (effort associated angina)
- Mild to moderate essential hypertension
- Obstructive hypertrophic cardiomyopathy
- Atrial fibrillation or flutter with rapid ventricular response
- Paroxysmal supraventricular tachycardia[8]
- Tarka™: mild to moderate essential hypertension

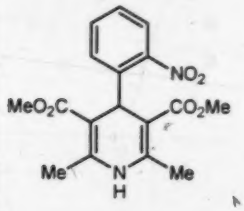
Pharmacology

- Inhibits calcium influx by blocking the voltage-dependent L-type calcium channel

[8] A sudden attack, recurrence or intensification of tachycardia located above the ventricles

Anti-Hypertensive 1,4-Dihydropyridine Calcium Channel Antagonists

Nifedipine



Therapeutic Indication

- Chronic stable angina (effort associated angina)
- Mild to moderate essential hypertension

Canadian Trade Name (Manufacturer)

- **Dosage Form**

Adalat (Bayer)

- Sustained-release oral tablet

Pharmacology

- Inhibits calcium influx by blocking the voltage-dependent L-type calcium channel

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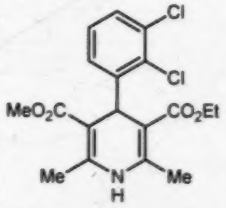
Felodipine



Therapeutic Indication

- Mild to moderate essential hypertension

Canadian Trade Name (Manufacturer)

- **Dosage Form**

Renedil (Aventis Pharma)

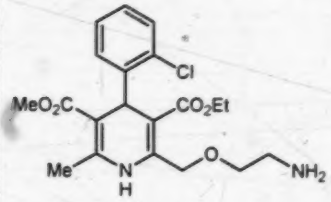
- Extended-release oral tablet

Pharmacology

- Inhibits calcium influx by blocking the voltage-dependent L-type calcium channel

---

Amlodipine



Therapeutic Indication

- Chronic stable angina (effort associated angina)
- Mild to moderate essential hypertension

Canadian Trade Name (Manufacturer)

- **Dosage Form**

Norvasc (Pfizer)

- Oral tablet

NB: Although nifedipine, felodipine and amlodipine all have once-a-day dosing, amlodipine does not require any specialized delivery system to extend its pharmacological effect.

Pharmacology

- Inhibits calcium influx by blocking the voltage-dependent L-type calcium channel

In the Prospective Randomized Amlodipine Survival Evaluation Study (PRAISE, 1996; 1153 patients), amlodipine seems to decrease mortality especially in non-ischemic cardiomyopathy patients. Further evaluation of the non-ischemic cardiomyopathy subgroup in PRAISE II suggests that although amlodipine is safe for these patients, the drug does not provide any tangible survival benefit [Packer *et al.*, 1996].

In the Vasodilator-Heart Failure Trial (V-HeFT III, 1997; 450 patients), felodipine showed no improvement in exercise capacity, or quality of life, or survival [Cohn *et al.*, 1997].

Thus, 1,4-dihydropyridine calcium channel antagonists should not be considered first line therapy for heart failure. Calcium channel antagonists should be used if additional control of blood pressure or cardiac afterload reduction is required or if other vasodilators are contraindicated [Ooi & Colucci, 2001].

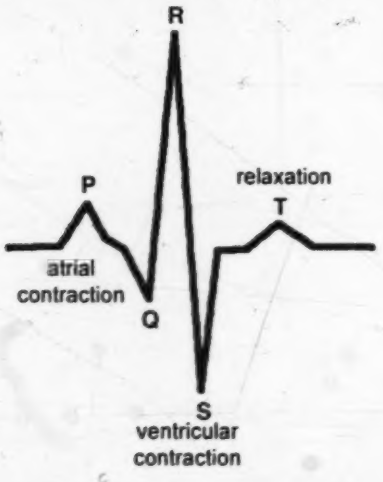


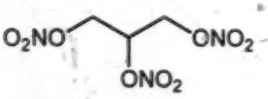
Figure 1.2.2: Electrocardiographic Intervals

Non-dihydropyridine class calcium channel antagonists have also been used as antiarrhythmics due to their ability to block calcium channels in the sinoatrial node and proximal portions of the atrioventricular node. Thus, these agents affect both heart rate and ventricular response. These class IV antiarrhythmics including verapamil, diltiazem and mibefradil are known to slow sinus rhythm and prolong atrial contraction (the PR interval) without affecting ventricular contraction (the QRS interval) [Hancox *et al.*, 2000] (Figure 1.2.2).

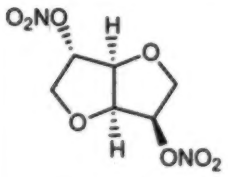
After its Food and Drug Administration (FDA) approval on June 20, 1997, mibefradil (Posicor™, Hoffman-La Roche) was promptly removed from the American market on June 8, 1998 [Willman, 2000]. Mibefradil when used in conjunction with various other drug classes was suspected of causing more than 143 sudden arrhythmia-related deaths in a 2400-patient study. Twenty-six drugs were known to be potentially dangerous if used with Posicor [FDA, 1998]. The FDA stated, "Since Posicor has not been shown to offer special benefits (such as treating patients who do not respond to other antihypertensive and anti-anginal drugs), the drug's problems are viewed as an unreasonable risk to consumers." Mibefradil was never introduced on the Canadian market.

1.2.3 Nitrovasodilators

Table 1.2.3: Nitrovasodilators Available in Canada

Glyceryl Trinitrate 	**Canadian Trade Name (Manufacturer)** - **Dosage Form**
Therapeutic Indication	NitroStat (Pfizer)
- Angina pectoris	- sublingual tablet
- Prophylaxis in situations likely to produce angina attack	Nitrolingual Spray (Aventis Pharma)
- Long-term prophylaxis for angina pectoris	- sublingual spray
- Patients with acute myocardial infarction on antihypertensives	NitroDur (Key)
	- transdermal patch
	Nitrol (Paladin)
	- ointment
Pharmacology	
- Prodrug for nitric oxide. Nitric oxide catalyzes the production of cyclic GMP.	Nitroglycerin in 5% Dextrose Injection (Baxter)
	- intravenous injection

Isosorbide Dinitrate



Canadian Trade Name (Manufacturer)

- **Dosage Form**

Apo-ISDN (Apotex)

- Sublingual or oral tablet

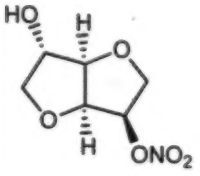
Therapeutic Indication

- Angina pectoris
- Prophylaxis in situations likely to produce angina attack
- Long-term prophylaxis for angina pectoris

Pharmacology

- Isosorbide dinitrate and its active metabolite isosorbide-5-mononitrate are both prodrugs for nitric oxide. Nitric oxide catalyzes the production of cyclic GMP.
- Isosorbide-2-mononitrate does not release a sufficient amount of nitric oxide to be therapeutically useful.

Isosorbide-5-Mononitrate



Canadian Trade Name (Manufacturer)

- **Dosage Form**

Imdur (AstraZenica)

- Extended-release oral tablet

Therapeutic Indication

- Angina pectoris
- Prophylaxis in situations likely to produce angina attack
- Long-term prophylaxis for angina pectoris

Pharmacology

- Active metabolite of isosorbide dinitrate that releases nitric oxide. Nitric oxide catalyzes the production of cyclic GMP.

Nitrovasodilators are very useful in treating patients with heart failure [CPhA, 2003] (Table 1.2.3). Nitrovasodilators increase peripheral venous capacitance and decrease pulmonary and systemic vascular resistance, thereby decreasing cardiac preload [Kevins & Robertson, 2001]. In simple terms, cardiac preload is the sum of forces needed to induce active forward injection of blood into the heart. Since the venous vasculature can move a larger volume of blood with less resistance, less energy is required to pump blood into the heart [Ooi & Colucci, 2001].

In fact, nitrovasodilators constitute a class of prodrugs that is denitrated to liberate nitric oxide. Nitric oxide relaxes bronchial, biliary, gastrointestinal, urethral and

uterine smooth muscle. As mentioned, nitric oxide exerts its favorable hemodynamic and anti-anginal action by vasodilating capacitance veins and conductive arteries. Vasodilated capacitance veins decrease ventricular volume and cardiac preload. To a lesser extent, a reduction in cardiac afterload is also caused by a decrease in left ventricular volume and systemic conductive arteries dilation. The overall result is a favorable effect on the imbalance between myocardial oxygen supply and demand in patients with coronary artery disease [CPhA, 2003].

Nitrovasodilators, when administered chronically to heart failure patients, improve exercise capacity and reduce symptoms [Johnson *et al.*, 1999].

Considering that myocardial infarction is the major cause of heart failure followed by hypertension [Francis, 2001], nitrovasodilators are especially beneficial in those patients with an underlying ischemic cardiomyopathy. Indeed, by increasing coronary blood flow to the epicardial coronary vasculature, nitrovasodilators enhance systolic and diastolic ventricular function [Ooi & Colucci, 2001].

However, patients on high doses of nitrovasodilators or those using nitrovasodilators with a long half-life may develop physiological tolerance to the action of nitric oxide. In those patients, nitrovasodilators become less efficacious, and the risk of experiencing side-effects such as migraine headaches, hallucinations and dementia are greatly increased. To prevent physiological tolerance to nitric oxide, patients on nitrovasodilators should be free from exposure to nitrovasodilators for 10 to 12 hours each day [CPhA, 2003].

Other than their use in cardiovascular disease such as relief of chest pain in angina pectoris and lower systemic blood pressure, nitrovasodilators may have a role in the treatment of cerebral vasospasm [Pluta *et al.*, 1997], male impotence [Heaton *et al.*, 1995], topical wound repair [Shabani, 1996], reverse pulmonary hypertension [Saavedra *et al.*, 1996; Hampl *et al.*, 1996; Brilli *et al.*, 1997], gastric ulceration associated with ingestion of non-steroidal anti-inflammatory agents [Gasco *et al.*, 1996; Wang *et al.*,2002], premature labor, fulminant liver failure[9], and sickle cell disease [Keefer, 1998]. Nitrovasodilators may also be used to treat bacterial [Wink *et al.*, 1995; Wink *et al.*, 1996; Donovan *et al.*, 1997] and parasitic [Rajan *et al.*, 1996; Ahmed *et al.*, 1997] infections as well as kill tumor cells [Mitchell *et al.*, 1993; Liebmann *et al.*, 1994]. Because nitric oxide prevents platelet self-adhesion and platelet adhesion to vascular

---

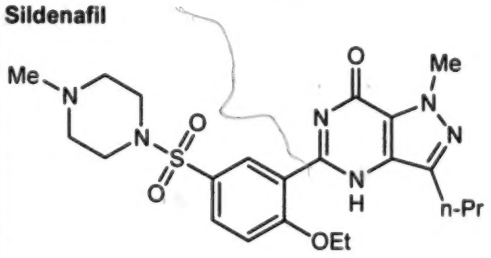
[9] Sudden or severe liver failure

implants, nitrovasodilators are also used to treat restenosis[10] following vascular surgery such as angioplasty, and to treat thrombosis [Maciejewski *et al.*, 1995; Kaul *et al.*, 1996; Chen *et al.*, 1997; Wang *et al.*, 2002], as well as other related diseases such as artherosclerosis [Kerwin *et al.*, 1995].

1.2.4 Cyclic GMP Specific Phosphodiesterase Type 5 Inhibitors

Table 1.2.4: Cyclic GMP Specific Phosphodiesterase Type 5 Inhibitors Available in Canada

Sildenafil



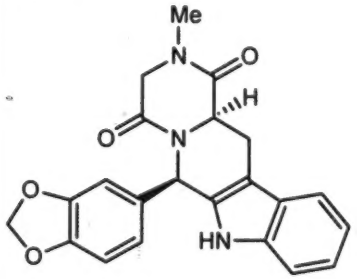
Canadian Trade Name (Manufacturer)
- **Dosage Form**

Viagra (Pfizer)
- oral tablet

Pharmacology
- Inhibits cyclic GMP specific phosphodiesterase type 5 which is responsible for the biodegradation of cyclic GMP

Therapeutic Indication
- male erectile dysfunction

Tadalafil



Canadian Trade Name (Manufacturer)
- **Dosage Form**

Cialis (Eli Lilly)
- oral tablet

Pharmacology
- Inhibits cyclic GMP specific phosphodiesterase type 5 which is responsible for the biodegradation of cyclic GMP

Therapeutic Indication
- male erectile dysfunction

[10] Renewed narrowing of a diseased blood vessel caused by tissue ingrowth and/or thrombosis following medical intervention to improve blood flow

Although related to the phosphodiesterase inhibitors used in the management of congestive heart failure, cyclic GMP specific phosphodiesterase type 5 (PDE5) inhibitors are more selective toward cyclic GMP than cyclic AMP (Table 1.2.4). PDE5 inhibitors are often used to potentiate the effect of intrinsic nitric oxide during sexual stimulation in men [CPhA, 2003].

In response to sexual stimulation, nitric oxide is released in the corpus cavernosum. Nitric oxide activates soluble guanylyl cyclase that then increases cyclic GMP levels leading to smooth muscle relaxation of the corpus cavernosum and allowing inflow of blood, thereby causing a sexual erection (Section 1.3.2) [Wang, 1994; Hellstrom, 1997].

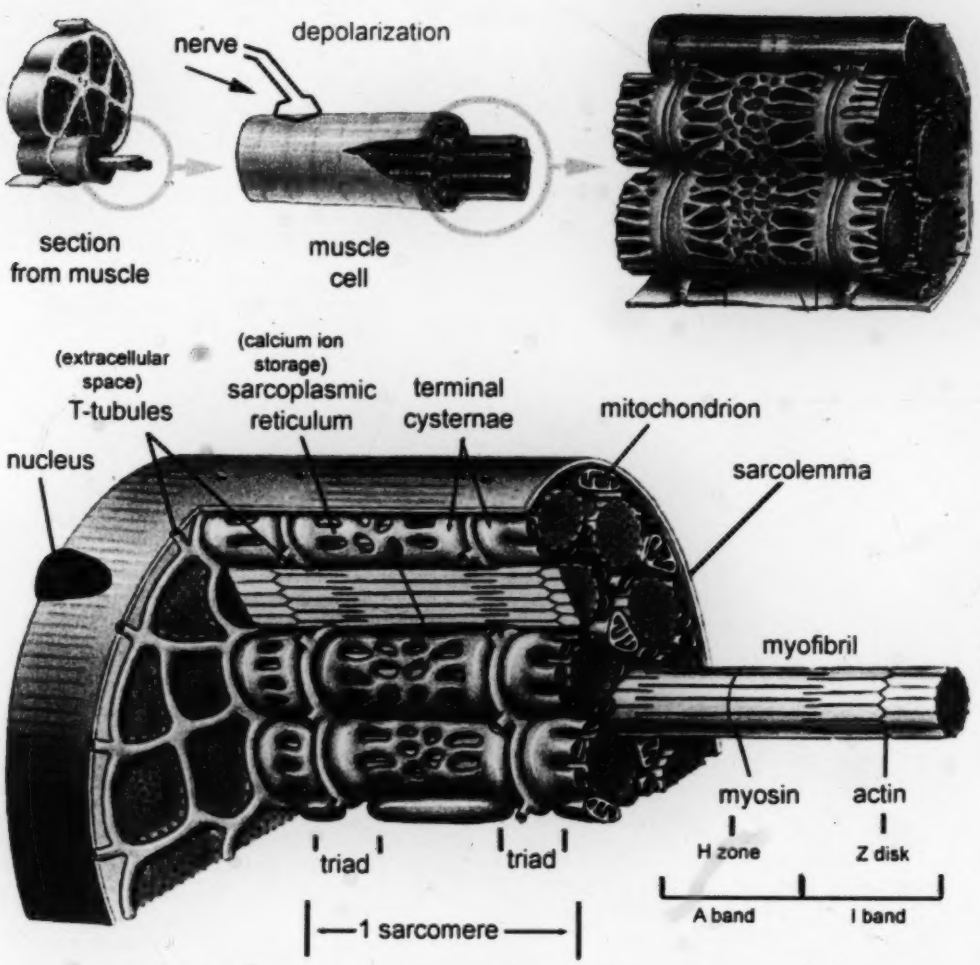
PDE5 inhibitors inhibit the PDE5 enzyme that is responsible for the biodegradation of cyclic GMP. Consequently, sexual erection is maintained for an extended period of time [CPhA, 2003].

1.3 MECHANISM OF ACTION

1.3.1 Calcium Channel Modulators

The calcium ion is the most fundamental factor involved in muscle contractility. The transmembrane entry of extracellular calcium ion through calcium channels is an important pathway for the excitation-contraction coupling process in the muscle [Fleckenstein, 1977; Triggle, 1992] (Figure 1.3.1a).

Increasing the intracellular calcium ion concentration increases the contraction of muscles. Calcium inflow is regulated by the calcium "leak" pathway [Zhorov *et al.*, 2001] and voltage-sensitive calcium channel receptor proteins [O'Rourke *et al.*, 1992]. In calcium homeostatis, intracellular calcium ion concentration is around 100 nM which is 10⁴ times below the extracellular calcium ion concentration [Egger & Niggli, 1999]. Because calcium is very toxic to cells, calcium outflow is regulated by the sodium/calcium ion exchangers [Egger & Niggli, 1999], calcium pumps [Muallem & Karlish, 1983], and ATP-depletion mechanisms [Hilgemann, 1997]. Distribution of intracellular calcium ion is regulated within the cell's organelles by calcium uptake and release processes in mitochondria [Petersen, 2002]; calcium release channels from the sarcoplasmic reticulum [Bezprozvanny & Ehrlich, 1993]; and calcium pumps to the sarcoplasmic reticulum [Bishop *et al.*, 1987].



Adapted from http://www.agen.ufl.edu/~chyn/age2062/lect/lect_19/179.gif and http://www.bmb.psu.edu/courses/bisci004a/muscle/musc-img/muscbig.jpg (accessed on October 29, 2004)

Figure 1.3.1a: Anatomy of a Skeletal Muscle Cell

Various contractile stimuli can produce an increase in intracellular calcium ion concentration. For example, an electrical current or an elevated potassium concentration depolarizes cellular membrane [Jones, 1998]. Depolarizing membrane stimuli increase calcium ion influx through voltage-sensitive channels at the muscular triads [Berridge, 1997]. An action potential travels along the nerve through the sarcolemmal T-tubules. There are voltage-sensitive proteins on the junctional T-tubule

membrane that are coupled with calcium release channels named ryanodine receptors on the junctional sarcoplasmic reticulum membrane at the terminal cysternae. The voltage-sensitive receptors in the T-tubule membrane respond to nerve stimulation by changing their conformations. The conformational change opens ryanodine receptors to release calcium ions from the sarcoplasmic reticulum to the cell. The influx of calcium into the cell opens other uncoupled ryanodine receptors, thus propagating the signal throughout the sarcoplasmic reticulum.

Another example is a direct drug-receptor interaction at the muscular triads. Uncoupled voltage-sensitive receptor protein act as calcium channels on the T-tubule membrane. Voltage-sensitive receptors are commonly referred to as "calcium channels" while ryanodine receptors are commonly referred to as "calcium release channels". A drug such as a calcium channel *agonist* increases intracellular calcium ion concentration. Conversely, a calcium channel *antagonist* prevents intracellular calcium ion concentration from increasing [Triggle, 1992].

Simply put, calcium channels in tissue exist as a mixture of opened and closed states known as "gating states". These calcium channels naturally switch between opened and closed states. When a calcium channel modulator binds to a calcium channel, the modulator either increases or decreases the rate of change. A calcium channel agonist prolongs the opened state of the calcium channel, thus allowing calcium ions to flow into the cell, thereby increasing the intracellular calcium ion concentration. A calcium channel antagonist prolongs the closed state of the calcium channel, thus slowing the influx of calcium ions into the cell, thereby preventing the intracellular calcium ion concentration from increasing. However, a calcium channel agonist can bind to a closed channel but will not alter the channel's state. Respectively, a calcium channel antagonist can also bind to an opened channel but will not alter the channel's state. Hence, the effect of a calcium channel modulator is affected by whether the channel is opened or closed [Triggle, 2002].

Calcium channel modulators also exhibit preferences in binding to a certain gating state. A calcium channel agonist exhibits little discrimination between opened and closed calcium channel state, whereas a calcium channel antagonist preferentially binds to channels that are closed [Triggle & Rampe, 1989].

As previously discussed, the effect of calcium channel modulators is affected by the gating state of the calcium channel. There are three modes of gating states for calcium channels. Mode 0 gating state has a greater percentage of closed channels

than opened channels, and is favored by calcium channel antagonists. Mode 1 gating state also has a greater population of closed channels but expresses brief bursts of channel openings. Mode 2 gating state has a large percentage of opened channels with brief bursts of closures, and is naturally favored by calcium channel agonists [Hess *et al.*, 1984].

A close-state calcium channel is not perfectly sealed against calcium ions. Both open- and close-state calcium channels permit calcium ions to enter into the cell. However, the rate of calcium ion inflow is much higher for open- than close-state calcium channels. Thus, by the calcium "leak" pathway, calcium ions can "leak" into the cell via calcium channels [Zhorov *et al.*, 2001].

Table 1.3.1a: The Properties of Voltage-Gated Calcium Channels

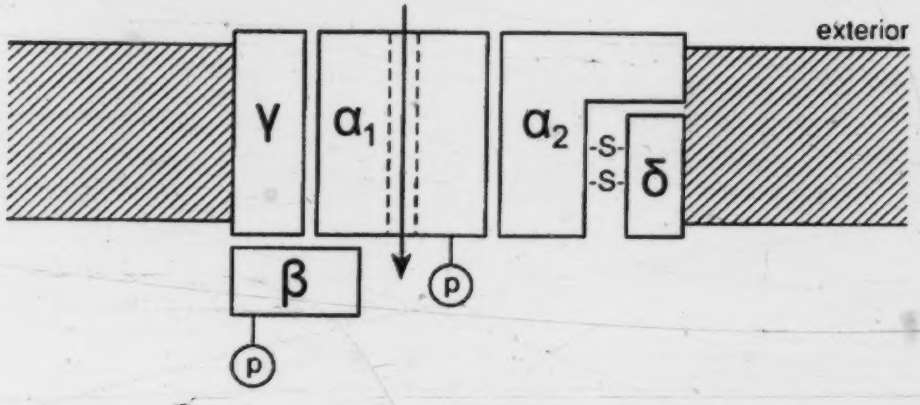
	L	T	N	P	Q	R
Conductance (pS)[11]	~ 25	~ 8	~ 10-20	~ 9-19	~ 16	N/A
Activation Threshold	high (> 30mV)	low (-70 to -50 mV)	high (> 30mV)			
Localization	cardiovascular system, endocrine cells, some neurons, smooth muscles		neurons			
Sensitivity to Drug Class	dihydropyridines (e.g. nifedipine), phenylalkylamines (e.g. verapamil), benzothiazepines (e.g. diltiazem), benzidimidazoles (e.g. mibefradil)	benzidimidazoles (e.g. mibefradil)	conotoxins and/or agatoxins			

Adapted from Triggle (1992, 2002)

Five major subtypes of voltage-sensitive calcium channels have been identified, namely N, P/Q, R, T and L. The N, P/Q and R types are high-voltage activated channels and are substantially localized to neurons. The T-type is a low-voltage activated channel

[11] ~100 mM Ba^{2+} as charge carrier

that is present in the cardiovascular, neuronal, endocrinal systems and in smooth muscles. Localized to similar regions as the T-type, the L-type is the most abundant and most voltage-sensitive channel of the group. Therapeutically useful calcium channel modulators (Table 1.2.2) only bind to the slow L-type calcium channels [Zamponi, 1997; Jones, 1998; Triggle, 2002] (Table 1.3.1a).



Adapted from Triggle (1992)

Figure 1.3.1b: The L-Type Calcium Channel

Table 1.3.1b: The Calcium Channel Subunits

	Number of Amino Acids	Molecular Weight (kD)	Transmembrane Domain	Function
α_1	~ 1873	~ 170	~ 24 (see Figure 1.3.1c)	Channel function: Voltage sensor and drug binding
α_2	~ 1106	~ 150	~ 3	Enhances α_1 function
β	~ 524	~ 55	~ 0	Cytoskeletal link. Possibly a peripheral protein
γ	~ 222	~ 32	~ 4	Only present in skeletal muscle
δ	~ 146	~ 17	unknown	Disulfide linked to α_2 subunit. Family of hydrophobic glycoproteins
Sum	~ 3871	~ 424		

Adapted from Triggle (1992)

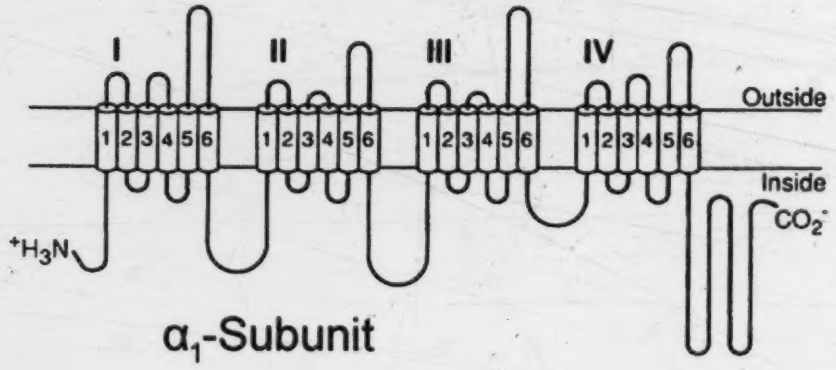
The calcium channel is composed of α_1, α_2, β, γ and δ subunits [Triggle 1992] (Figure 1.3.1b). The α_1-subunit is responsible for channel function including voltage sensing and drug binding, while the α_2 and β subunits enhance co-expression of channel function. Calcium channel modulators bind to the large, pore-forming α_1-subunit [Triggle, 1992; Triggle, 2002] (Table 1.3.1b).

Table 1.3.1c: The Properties of Calcium Channel α_1-Subunits

Current Name	Former Name	Tissue	Channel Type	Sensitivity to Drug Class
$Ca_v1.1$	α_{1S}	skeletal muscle	L	dihydropyridines (e.g. nifedipine), phenylalkylamines (e.g. verapamil), benzothiazepines (e.g. diltiazem), benzidimidazoles (e.g. mibefradil)
$Ca_v1.2$	α_{1C}	smooth muscle, heart, brain		
$Ca_v1.3$	α_{1D}	brain, glands		
$Ca_v1.4$	α_{1F}	retina		
$Ca_v2.1$	α_{1A}	brain, pituitary gland	P/Q, N and R	conotoxins and/or agatoxins
$Ca_v2.2$	α_{1B}	neurons		
$Ca_v2.3$	α_{1E}	brain, pituitary gland		
$Ca_v3.1$	α_{1G}	neurons	T	benzidimidazoles (e.g. mibefradil)
$Ca_v3.2$	α_{1H}	brain, heart, kidney, liver		
$Ca_v3.3$	α_{1I}	brain		

Adapted from Triggle (1992, 2002)

There are a variety of α_1-subunit types. Therapeutically useful calcium channel modulators (Table 1.2,2) bind to $Ca_v1.1$ in skeletal muscle; $Ca_v1.2$ in smooth muscle, heart and brain; $Ca_v1.3$ in brain and glands; and $Ca_v1.4$ in the retina [Triggle, 1992; Triggle 2002] (Table 1.3.1c).



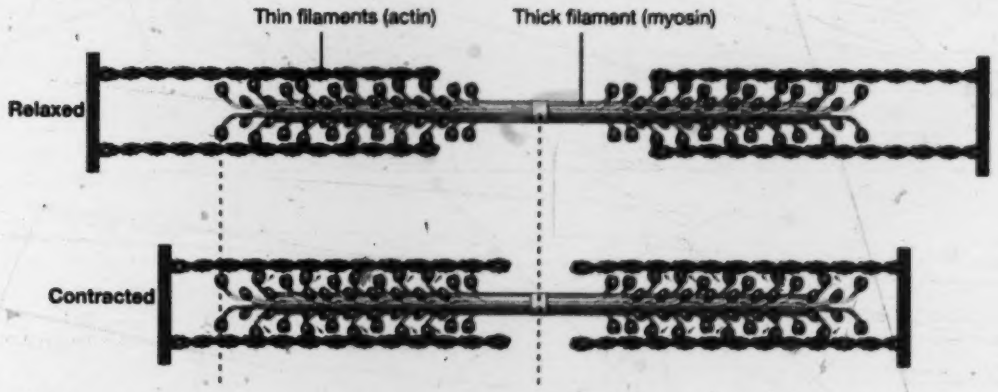
Adapted from Triggle (1992)

Figure 1.3.1c: The α_1-Subunit

The α_1-subunit is composed of four homologous domains: I, II, III and IV [Triggle, 1992] (Figure 1.3.1c). Each domain is composed of six transmembrane segments: S1 – S6. Phenylalkylamine calcium channel antagonists such as verapamil bind to IVS6. Benzothiazepine calcium channel antagonists such as diltiazem bind to the cytoplasmic bridge between III and IV. The 1,4-dihydropyridine calcium channel modulators bind to IIIS6 and IVS6. Hence, because the α_1-subunit can accommodate three different drug classes, these separate domains are believed to have allosteric interaction [Triggle, 1992; Mitterdorfer *et al.*, 1998]. Moreover, when administered simultaneously 1,4-dihydropyridine agonists and antagonists compete for receptor sites since both bind to the same transmembrane segments [Janis & Triggle, 1984; Su *et al.*, 1984].

In cardiac and skeletal muscles, increasing intracellular calcium ion enhances binding of calcium ion to a protein called, "troponin". Once bound, troponin dislodges from between actin and myosin. Actin and myosin interact and cause contraction [Natale, 1999; Kevins & Robertson, 2001] (Figure 1.3.1d).

In vascular smooth muscles, increasing intracellular calcium ion enhances binding of calcium ion to a protein called, "calmodulin". The calcium ion-calmodulin complex activates myosin light-chain kinase. The kinase then phosphorylates the light chain of myosin. Phosphorylation promotes interaction between actin and myosin resulting in muscular contraction [Natale, 1999; Kevins & Robertson, 2001].



Adapted from http://fig.cox.miami.edu/~cmallery/150/neuro/sf43x16.jpg (accessed on October 29, 2004)

Figure 1.3.1d: Relaxed and Contracted States of Actin-Myosin Complexes

1,4-Dihydropyridine activity is potential dependent [Janis & Triggle, 1984]. At different membrane-holding potentials, a 1,4-dihydropyridine calcium channel agonist may switch its pharmacological properties from an agonist to an antagonist. In cardiac Purkinje fibers of calves, the 1,4-dihydropyridine calcium channel agonist BAY K 8644 (Figure 1.4.1.1.2c) acts as an agonist at membrane-holding potentials less than -50 mV, but as an antagonist at less than -45 mV [Sanguinetti & Kass, 1984a]. Conversely, 1,4-dihydropyridine calcium channel antagonists show little antagonist activity at membrane-holding potentials less than -60 mV, however, substantial activity is expressed at less than -45 mV [Sanguinetti & Kass, 1984b]. Thus, these studies suggest that a 1,4-dihydropyridine calcium channel modulator behaves as an agonist at -50 mV and as an antagonist at -45 mV. It is a possibility that most calcium channels are opened at -50 mV, and that most calcium channels are closed at -45 mV. Moreover, the fact that a calcium channel agonist exhibits little discrimination between opened and closed calcium channel state, whereas a calcium channel antagonist preferentially binds to channels that are closed [Triggle & Rampe, 1989] suggests that a calcium channel agonist is more likely to switch pharmacological properties than a calcium channel antagonist which is more likely to lose pharmacological properties.

Interestingly, at very high concentrations, a 1,4-dihydropyridine calcium channel agonist can behave as an antagonist. Essentially, when the tissue is saturated with a calcium channel agonist, most of the calcium channels are bound by the agonist.

Because most channels are opened, the change in calcium ion influx rate decreases; in other words, the resting membrane potential becomes lower. A low resting membrane potential does not induce muscular contraction. Thus, in this case, when compared to the state when calcium channel agonist concentration is optimal, the calcium channel agonist behaves as a calcium channel antagonist. In this regard, as the channels become saturated, the agonist effect is decreased [Holland *et al.*, 1989; Triggle & Rampe, 1989; Gross *et al.*, 1990].

Conversely, at very low concentrations, a 1,4-dihydropyridine calcium channel antagonist can behave as an agonist. When the tissue is in a relaxed state, resting membrane potential is average. When a very low concentration of the antagonist is introduced, the antagonists induce the few closed calcium channels to remain closed. This prolongation of the closed state causes a change in the calcium ion influx rate; in other words, the resting membrane potential becomes higher. A high resting membrane potential induces muscular contraction, and thus, the calcium channel antagonist behaves as a calcium channel agonist [Holland *et al.*, 1989; Triggle & Rampe, 1989; Gross *et al.*, 1990].

1.3.2 Nitrovasodilators

Similar to the end-mechanism of action of calcium channel modulators on vascular smooth muscles, phosphorylation of the myosin light chain in cardiac and smooth muscles regulates the maintenance of the contractile state in cardiac and vascular smooth muscles. Nitrovasodilators indirectly dephosphorylate the myosin light chain to induce muscle relaxation, thus leading to vasodilation [Ooi & Colucci, 2001].

Throughout the vasculature, during the conversion of arginine to citrulline, nitric oxide is produced [Kerwin *et al.*, 1995]. Arginine conversion and the release of nitric oxide are catalyzed by nitric oxide synthases bound to either troponin or calmodulin (Table 1.3.2). Nitric oxide is an intracellular and paracrine signaling autocoid [Kevins & Robertson, 2001] that is also known as an endothelium-derived relaxing factor [Palmer *et al.*, 1987]. Nitric oxide is a reactive, free radical that activates soluble guanylyl cyclase [Kevins & Robertson, 2001]. The activated soluble guanylyl cyclase then increases the synthesis of cyclic GMP in cardiac and smooth muscles. Cyclic GMP activates a protein kinase. The protein kinase then breaks down the phosphorylation of

various proteins. One of these proteins, the light chain of myosin, is dephosphorylated and muscle relaxation occurs.

Nitrovasodilators release nitric oxide. The nitrovasodilator nitroprusside spontaneously converts to nitric oxide in the presence of reducing agents such as glutathione. Nitrovasodilators such as glyceryl trinitrate are converted to nitric oxide or bioactive S-nitrosothiols by a more complex enzymatic biotransformation [Kevins & Robertson, 2001].

Table 1.3.2: The Nitric Oxide Synthases

Nitric Oxide Synthase Isozyme	Number of Amino Acids	Molecular Weight (kD)	Expression	Pharmacology
Endothelial (eNOS)	~ 1433	~ 160	Constitutive	- Phosphorylated upon stimulation of endothelial cells by shear stress, inflammatory mediators, bradykinin and other vasorelaxant agonists
Neuronal (nNOS)	~ 1153	~ 130	Constitutive	- In central nervous system, activity is associated with nitric oxide-mediated synaptic plasticity.[12] - In peripheral nervous system, activity is associated with smooth muscle relaxation.
Inducible (iNOS)	~ 1203	~ 133	Requires cytokine induction	- Induced by various inflammatory stimuli (cytokines) - Unlike the other isozymes, the regulation of its activity is not dependent on calcium ion influx

Adapted from Triggle (2002) and Kerwin *et al.* (1995)

Nitric oxide also affects other physiological systems and acts as a mediator of immune system function, neurotransmition and anticoagulation.

[12] Formation and maintenance of neurons

As a mediator of immune system function, nitric oxide is known to retard the proliferation of many cell types including smooth muscle cells and certain nitric oxide-sensitive types of cancer cells [Saavedra *et al.*, 2000], and to mediate many of the immune system's antibacterial [Pacelli *et al.*, 1996; Wink *et al.*, 1995; Wink *et al.*, 1996; Donovan *et al.*, 1997], antiparasitic [Rajan *et al.*, 1996; Ahmed *et al.*, 1997], antitumor [Mitchell *et al.*, 1993; Liebmann *et al.*, 1994], antiviral and antifungal effects [Keefer, 1998]. Indeed, at inflamed and damaged tissue sites, nitric oxide is released by macrophages to retard cellular development of tumor, bacterial, viral and fungal cells [Marletta *et al.*, 1988].

In the peripheral system, as a neurotransmitter, nitric oxide affects nonadrenergic and noncholinergic nerves to promote gastrointestinal motility [Gasco *et al.*, 1996; Wang *et al.*,2002], and dilates airways of the lungs [Saavedra *et al.*, 1996; Hampl *et al.*, 1996; Brilli *et al.*, 1997]. Moreover, as a paracrine autocoid, nitric oxide mediates cerebral intercellular communication such as learning and memory by coupling neuronal activity to cerebral blood flow [Pluta *et al.*, 1997] and by facilitating the release of several neurotransmitters and hormones [Gasco *et al.*, 1996].

Nitric oxide also inhibits the adhesion, aggregation and activation of platelets [Maciejewski *et al.*, 1995; Kaul *et al.*, 1996; Chen *et al.*, 1997; Wang *et al.*, 2002], and attenuates leukocytes adherence and activation [Kerwin *et al.*, 1995].

Other than its role in maintaining macrovascular homeostatis, nitric oxide also contributes to the maintenance of microvascular homeostatis. Indeed, in men, nitric oxide also stimulates penile erection by initiating flow of blood to the penis [Heaton *et al.*, 1995], while in women, nitric oxide relaxes the uterus during pregnancy [Keefer, 1998].

However, the overproduction of nitric oxide *in vivo* often leads to different pathological states such as migraine headaches, hallucinations and dementia [CPhA, 2003]. Therefore, many nitric oxide synthase inhibitors are currently under investigation to regulate the excessive formation of nitric oxide. L-N^G-methylarginine (NMA)[13] and L-N^G-nitroarginine methyl ester (NAME) are classical examples of non-selective nitric oxide synthase inhibitors. Other examples of nitric oxide synthase inhibitors include L-N^G-nitroarginine, L-N^G-aminoarginine, L-iminoethylornithine, L-iminoethyllysine, L-N^G-hydroxy-N^G-methylarginine, L-N^G-methyl-N^G-methylarginine, L-N^G-methyl-$N^{G'}$-methylarginine, L-thiocitrulline, L-*S*-methylisothiocitrulline, L-*S*-ethylisothiocitrulline, *S*-

[13] "N^G" denotes "nitrogen that interacts with guanylyl"

ethylisothiourea, *S,S'*-(1,3-phenylenebis(1,2-ethanediyl)bis isothiourea, some of which are more selective toward certain nitric oxide synthase isozymes [Kerwin *et al.*, 1995].

1.4 1,4-DIHYDROPYRIDINE CALCIUM CHANNEL MODULATORS

1.4.1 Quantitative Structure-Activity Relationships

The development of useful structure-activity relationships from physical, chemical and biologic experimental data facilitates the design of compounds that have targeted calcium channel modulation properties. Physicochemical and biologic data are gathered from multiple sources using several methods, examined, manipulated, correlated, and re-examined in an effort to find relating characteristics. Groups of compounds having those characteristics are then designed, synthesized, and subjected to various pharmacological experiments. Pharmacological data are then carefully examined, manipulated and correlated to add to the current body of knowledge. From this body of knowledge, additional classes of compounds can be prepared for biological evaluation.

$$\text{Activity} = k_1\,(\text{molecular size}) + k_2\,(\text{molecular weight}) + k_3\,(\text{hydrophobicity}) + k_4\,(\text{degree of ionization}) + k_5$$

Figure 1.4.1a: An Example of Property-Activity Relationships

Several methods have been designed to identify correlations between molecular properties and pharmacologic activity. The Property-Activity Relationships method seeks a correlation with the molecule's physicochemical properties such as molecular size, molecular weight, hydrophobicity and degree of ionization (Figure 1.4.1a). Because the molecule is examined as a whole, this method is most useful for simple molecules or those molecules that have a simple relationship with pharmacological activity.

$$\text{Activity} = k_1\,(\text{activity at functional group 1}) + k_2\,(\text{activity at functional group 2}) + k_3\,(\text{activity at functional group 3}) + k_4$$

Figure 1.4.1b: An Example of Property-Intrinsic Activity Relationships

An elaboration of the linear Property-Activity Relationship method leads to a two-dimensional method in which each region of the molecule is individually examined and correlated with pharmacologic activity (Figure 1.4.1b). In the Property-Intrinsic Activity Relationship equation, the definition of the "activity at functional group" parameters is often the sum of the functional group's physicochemical properties. In simpler words, Property-Activity Relationship equations at each functional group are tabulated to form a larger structure-activity relationship equation.

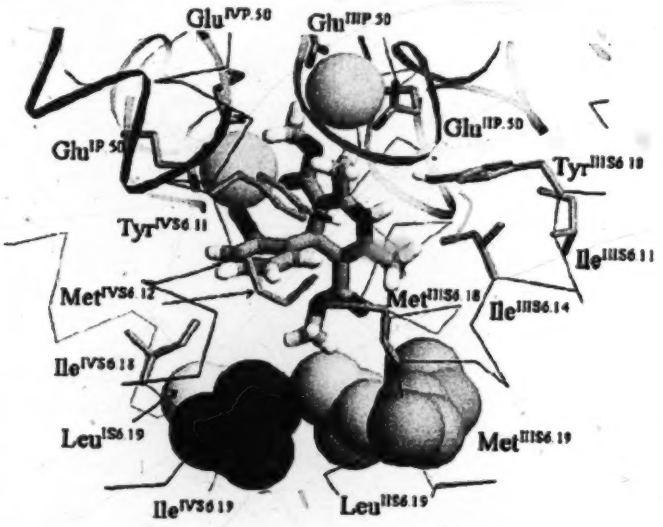
To test the reliability of a structure-activity relationship equation various statistical methods are employed. The most fundamental requirement is that the total number of parameters found in the equation should be less than the number of data from which the equation is derived. Ideally, the equation should have the least number of parameters for the equation to be considered reliable. For that reason, each intrinsic activity at each functional group is represented as a single parameter in the Property-Intrinsic Activity Relationship equation (Figure 1.4.1b).

When it comes to 1,4-dihydropyridine calcium channel modulators, structure-activity relationships analysis is difficult because of the problem of multicollinearity of substituent parameters, a high-leverage point, and position-dependent grouped observations [Mager *et al.*, 1992].

The term "multicollinearity of substituent parameters" implies that the activity of a functional group may be interdependent with the activities of other functional groups that are present on the molecule. Indeed, multicollinearity is especially apparent for small molecules such as 1,4-dihydropyridines with functional groups that are close to each other.

In a Property-Intrinsic Activity Relationship equation (Figure 1.4.1b), if the activity of each functional group is restricted to a common range, the significance of each parameter is determined by the constants k_n. Many separate studies have been reported for 1,4-dihydropyridines that have specific functional groups at specific positions. For each group, structure-activity relationship equations have been derived with different significance assigned to each parameter. When compared from one study to another, the significance of each parameter varies greatly, which suggests that high-leverage points and outliers yield false significance or insignificance. In simpler words, an equation with a high leverage point places too much dependency on a specific parameter. Conversely, an equation's outlier is a parameter that contributes significantly to a specific series, but contributes insignificantly to an equation containing all possible

series. If all the equations from each study are combined into a very large equation, the number of parameters is too great to make the overall equation reliable.



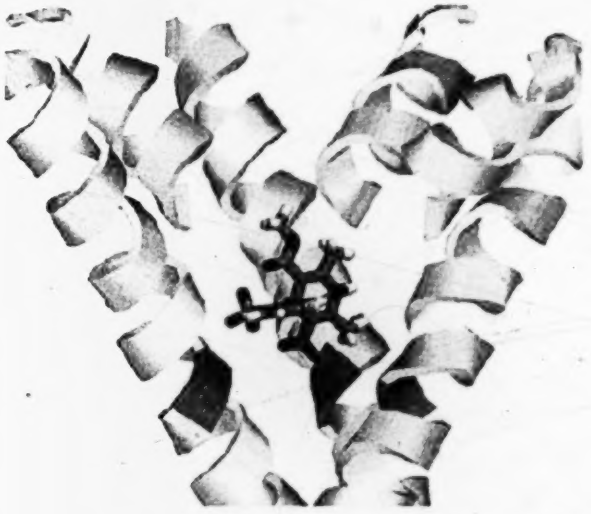
Adapted from Zhorov *et al.* (2001)

Figure 1.4.1c: A Close-Up View of Nifedipine Docked in a Pseudo α_1-Subunit of the Calcium Channel Receptor

A further elaboration of the Property-Intrinsic Activity Relationships method leads to computer graphics simulations [Zhorov *et al.*, 2001] (Figure 1.4.1c). A molecule is modeled and its coordinates are optimally energy minimized. A receptor is modeled according to X-ray crystallographic or NMR data, and its coordinates are also optimally energy minimized. The molecule is then placed inside of the receptor and the molecule-receptor system subjected to dynamic energy minimization. Molecule-receptor stability is determined by theoretical van der Waals interactions and electronegative bonding-energy estimations. To represent the three-dimensional aspect of the model, the result is often expressed in terms of coordinates and charges between the atoms of the molecule and receptor.

To date, the calcium channel protein has not been adequately purified such that X-ray crystallographic or NMR data can accurately describe the coordinates of the calcium channel L-type receptor protein. However, because voltage-gated calcium channels share similar transmembrane topology with sodium and potassium channels, it is possible to modify the known coordinates for the sodium or potassium channel to

mimic a calcium channel. The sodium and calcium channels have four homologous domains arranged quasi-symmetrically around the central pore forming the α_1-subunit [Zhorov *et al.*, 2001]. The potassium channel's α_1-subunit is composed of four identical domains arranged symmetrically around the central pore forming an inverted teepee motif. X-Ray crystallographic data for a potassium channel KcsA was obtained and the potassium channel was modeled [Doyle *et al.*, 1998]. Zhorov *et al.* (2001) and Zamponi *et al.* (2003) have both modified the potassium channel coordinates in an attempt to apply them to the calcium channel protein (Figure 1.4.1d). In this regard, the authors have changed potassium channel's amino acids to those of calcium channel amino acids that are present in the calcium channel receptor site that bind to the 1,4-dihydropyridine drug. The potassium channel's unchanged amino acids are retained to serve as a scaffold. The amino acid numbering sequence is different in each study, because each research group used different methods for amino acid segment alignment and also different calcium channel fragment data.



Adapted from Zhorov *et al.* (2001)

Figure 1.4.1d: Nifedipine Docked in a Pseudo α_1-Subunit of the Calcium Channel Receptor

Because computer graphics simulations are done on a modified potassium channel and not a true calcium channel, and because the potassium channel's α_1-subunit is much smaller (77 kDa) relative to that of the larger calcium channel (170 kDa)

[Triggle, 1992; Doyle *et al.*, 1998], the Property-Intrinsic Activity Relationship method is still the predominant driving force used to design 1,4-dihydropyridine modulators. Although 1,4-dihydropyridine calcium channel modulators are currently not considered as first line therapy for heart failure [Ooi & Colucci, 2001], novel 1,4-dihydropyridines, that are derived from quantitative structure-activity relationships, could take advantage of previously synthesized 1,4-dihydropyridines' proven desired pharmacological effects while avoiding the undesired pharmacological effects.

1.4.1.1 The Central Ring

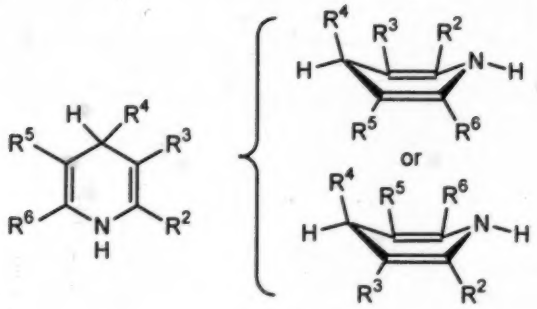


Figure 1.4.1.1a: Pharmacophore for 1,4-Dihydropyridines

1,4-dihydropyridine calcium channel modulators have a 1,4-dihydropyridine ring that exists in a flattened pseudo-boat conformation [Langs *et al.*, 1991; Triggle, 1992] (Figure 1.4.1.1a). The R^4 substituent is located above the "boat" and is usually an aromatic or heteroaromatic moiety. The proton attached to C-4 is located below the "boat" [Baldwin *et al.*, 1987; Triggle, 1992; Ochoa *et al.*, 1998]. The C-4 carbon is a chiral centre where the molecule's *S* or *R* configuration is determined by the R^3 and R^5 substituents [Mahmoudian & Richards, 1986a; Triggle & Rampe, 1989; Triggle, 1992], since the proton and R^4 at C-4 have a restricted configuration. Potent calcium channel antagonists usually have esters as R^3 and R^5 substituents [Kappe & Fabian, 1997], whereas potent calcium channel agonists usually have a nitro moiety as R^3 and an ester as R^5 substituents [Triggle, 1992]. Most calcium channel modulators have methyl moieties as R^2 and R^6 substituents [Bossert & Vater, 1989]. A secondary amine is required for pharmacologic activity [Fossheim *et al.*, 1982; Fossheim, 1987; Langs *et al.*, 1991; Triggle, 1992; Kappe & Fabian, 1997; Natale *et al.*, 1999].

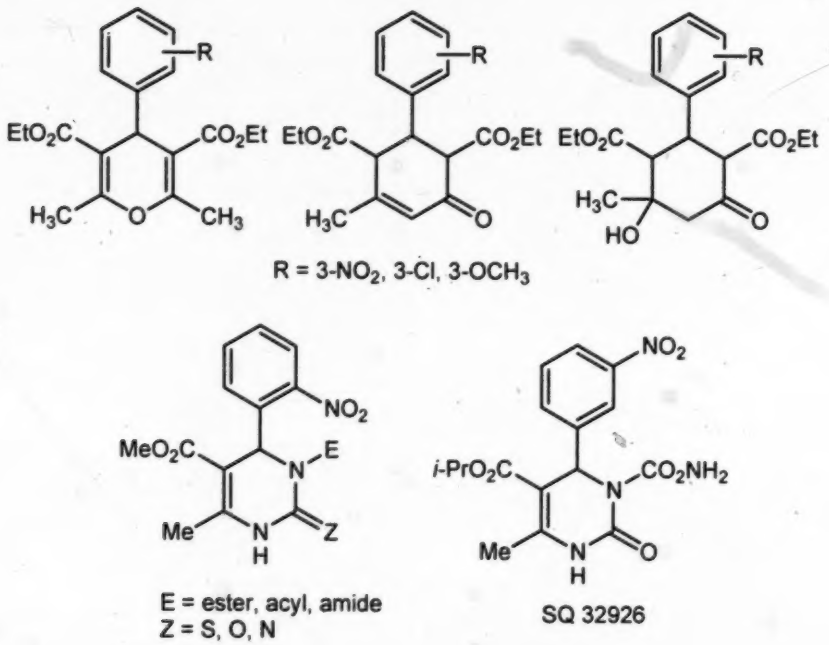


Figure 1.4.1.1b: Analogues of 1,4-Dihydropyridines

Although much weaker in activity, pyran derivatives, cyclohexanone derivatives and *N*-substituted 1,4-dihydropyridines have demonstrated that calcium channel antagonist activity is not limited to the 1,4-dihydropyridine drug class [Bossert & Vater, 1989] (Figure 1.4.1.1b). Dihydropyrimidines and other 3-aza-analogues of dihydropyridines show a similar pharmacological profile to those of dihydropyridines as calcium channel modulators [Kappe & Fabian, 1997]. In this regard, the 1,4-dihydropyrimidine, SQ32926, has an equipotent calcium channel antagonist activity compared to related 1,4-dihydropyridine diester analogues.

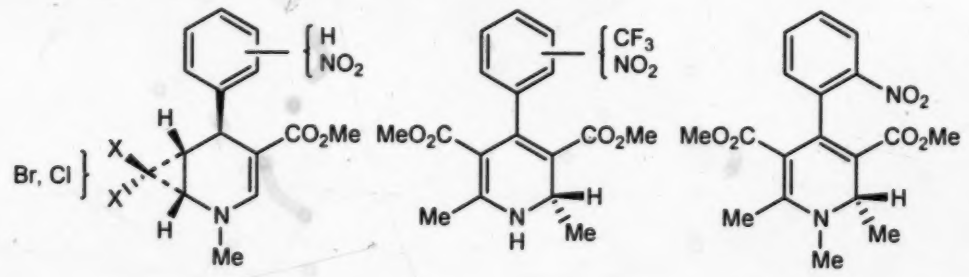


Figure 1.4.1.1c: 1,4-Dihydropyridines Analogues Prepared in Our Research Group

Studies carried out in our research group involving dihalocyclopropyl and 1,2-dihydropyridinyl analogues as potential bioisosteres for the 1,4-dihydropyridine ring did not show any improvement in activity relative to 1,4-dihydropyridine rings [Wynn *et al.*, 1988; Moharram *et al.*, 2004] (Figure 1.4.1.1c).

1.4.1.2 The C-4 Aromatic Substituent

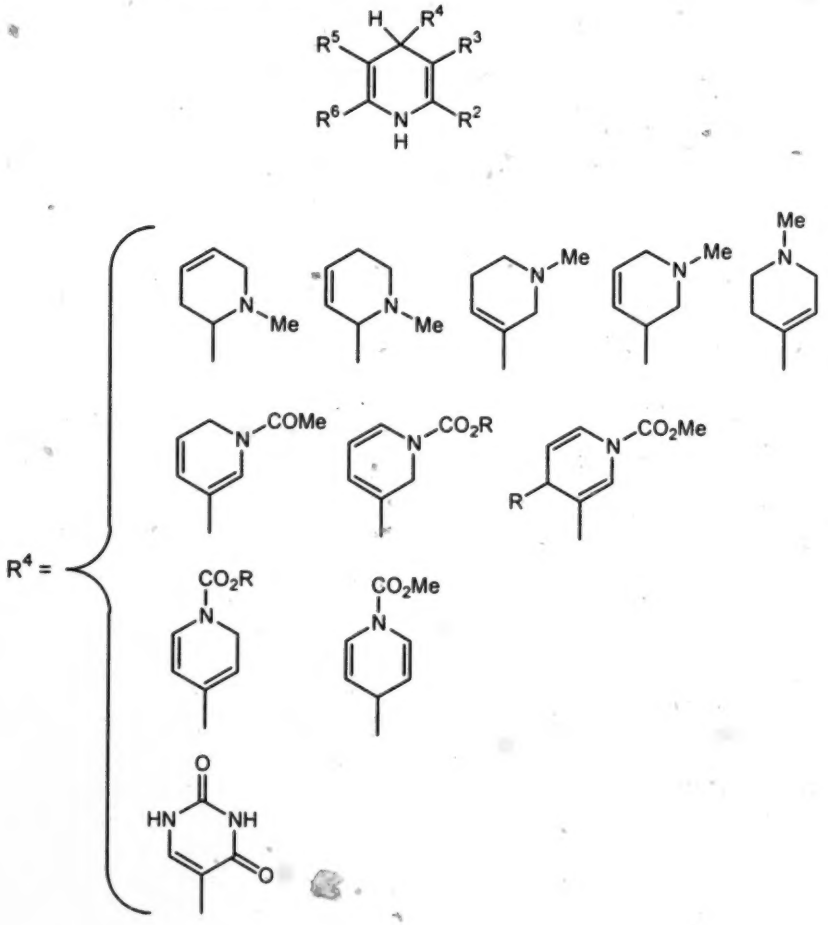


Figure 1.4.1.2a: Non-Aromatic 1,4-Dihydropyridines

For calcium channel modulator activity, one aromatic group (R^4) is attached to the C-4 position that is positioned above the 1,4-dihydropyridine "boat" [Baldwin *et al.*,

1987; Triggle, 1992; Ochoa *et al.*, 1998]. This aryl ring, which binds to a flat region on the receptor, influences the extent of puckering for the 1,4-dihydropyridine ring [Fossheim, 1987]. According to computer modeling and X-ray crystallographic data, a less puckered "boat" 1,4-dihydropyridine ring is more likely to exhibit more potent calcium channel agonist than antagonist activity, and vice versa [Fossheim *et al.*, 1982; Fossheim *et al.*, 1986; Mager *et al.*, 1992; Rovnyak *et al.*, 1995]. The extent of 1,4-dihydropyridine ring puckering, being the central region of the molecule, affects the coordinates of attached functional groups, thus altering the proximal distances between the molecule and the receptor surface.

Our research group has synthesized several classes of 1,4-dihydropyridines where the R^4 substituents are non-aromatic moieties [Dagnino *et al.*, 1987a; Dagnino *et al.*, 1987b; Ramesh *et al.*, 1992; Ramesh *et al.*, 1999; Amini *et al.*, 2001; Fassihi *et al.*, 2004] (Figure 1.4.1.2a). For calcium channel antagonist activity, the effect of these classes of compounds on guinea pig ileal longitudinal smooth muscle ranged from inactive to moderately active ($IC_{50} = 3.1 \times 10^{-8}$ M) relative to nifedipine ($IC_{50} = 1.4 \times 10^{-8}$ M). For calcium channel agonist activity, their effect on guinea pig left atria ranged from inactive to moderately active ($EC_{50} = 2.91 \times 10^{-6}$ M) relative to BAY K 8644 ($EC_{50} = 7.7 \times 10^{-7}$ M).

For subtituents on the aryl groups (R^4), optimum antagonist activity is reported as **ortho ≥ meta >> para**, that electron withdrawing aryls are more potent than electron releasing aryls, and that activity is dependent on the size of the aryl group [Fossheim *et al.*, 1982; Bossert & Vater, 1989; Mager *et al.*, 1992; Triggle, 1992]. For good antagonist activity, meta substituents should be wide but not lengthy, while para substituents should be small [Mahmoudian & Richards, 1986b].

Furthermore, the environment around the ortho-position of the aryl group (R^4) attached at C-4, is believed to be lipophilic, transitioning to a more hydrophilic environment around the meta-position, and becoming hydrophilic at the para-position [Mahmoudian & Richards, 1986b; Natale *et al.*, 1999; Zamponi *et al.*, 2003] (Figure 1.4.1.2b). Two phenylalanine residues at the IIIS6 portion of the receptor form a lipophilic pocket around the aryl group (R^4). In between these two phenylalamine residues, there is a tyrosine residue at the IVS6 portion of the receptor. Hence, the aryl group (R^4) is flanked by two phenylalanine residues that contribute to a lipophilic environment at the ortho-position. Moreover, a para-positioned substituent may act as a hydrogen-bond acceptor with the tyrosine residue [Natale *et al.*, 1999]. However, for

1,4-dihydropyridine calcium channel agonists, para-substitutents on the aryl group (R^4) greatly reduce agonist activity [Triggle & Rampe, 1989].

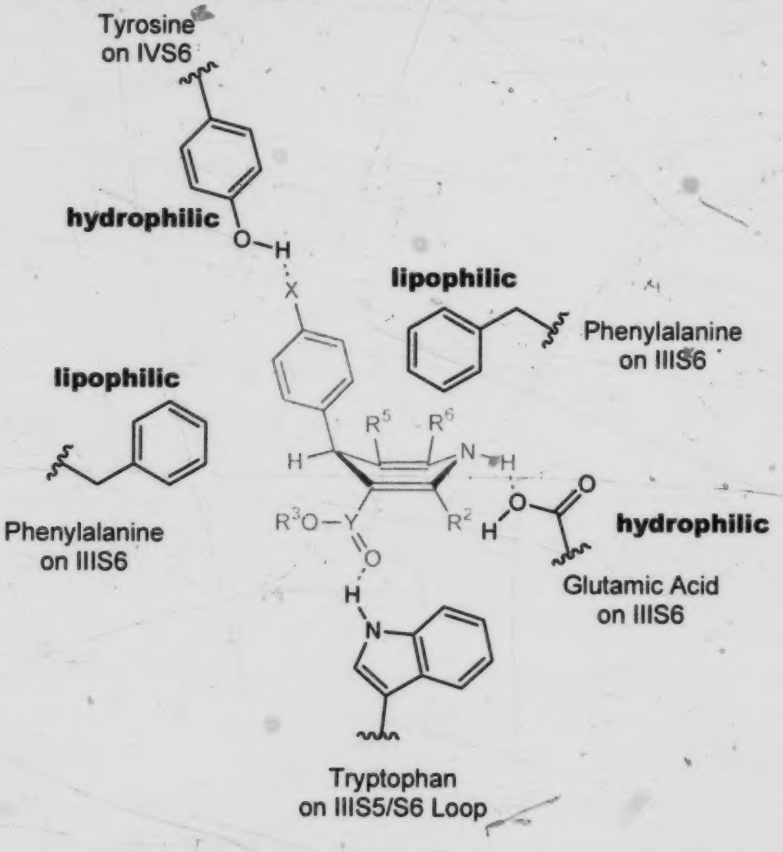


Figure 1.4.1.2b: First Model – Proposed 1,4-Dihydropyridine Receptor Surface Binding Site

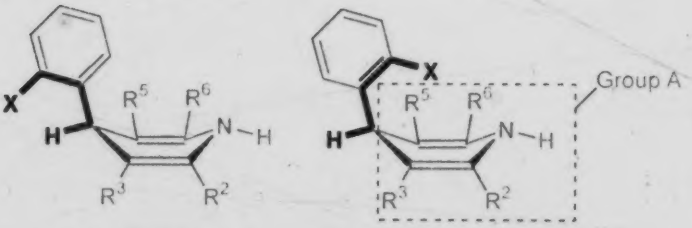


Figure 1.4.1.2c: 1,4-Dihydropyridines Possessing Aryl *Syn* and *Anti* Rotameric Orientations

There is a somewhat restrictive rotation about the bond between the C-4 carbon and the atom to which the aromatic substituent is attached. There are two major rotameric orientations for the aromatic substituent attached at C-4. Aryl ring torsion angle is affected by whether the aromatic substituent is positioned on the same side as the C-4 hydrogen (aryl *syn* rotamer) or away from the C-4 hydrogen (aryl *anti* rotamer) (Figure 1.4.1.2c). "*Syn*" and "*anti*" are defined as the relative orientation of the C-4 hydrogen and the ortho-substituted X on the aromatic functional group with respect to the bond connecting the 1,4-dihydropyridine C-4 carbon and C-1 of the aryl substituent. Most 1,4-dihydropyridines assume a slightly higher ratio of *syn* relative to *anti* rotameric orientation in solution [Goldmann & Geiger, 1984; Rovnyak *et al.*, 1988]. Moreover, the *syn* rotamer is preferred at the receptor [Rovnyak *et al.*, 1991; Rovnyak *et al.*, 1995]. Steric hindrance is a plausible explanation for the preferred *syn* rotamer, because the *syn* orientation is only hindered by the C-4 proton whereas the *anti* orientation is more sterically hindered by Group A (Figure 1.4.1.2c) [Rovnyak *et al.*, 1991, Parrish *et al.*, 2000].

When compared to a compound containing a *small* ortho *syn* aryl substituent, a compound containing a *bulky* ortho *syn* aryl substituent has a more obtuse angle between the C-4 hydrogen, C-4 carbon and C-1 of the aryl substituent [Mahmoudian & Richards, 1986b; Rovnyak *et al.*, 1988]. This obtuse angle increases the distance between atom X and C-4 hydrogen to relieve steric strain. As a result, the "boat" is more puckered, and such a compound is a very potent calcium channel antagonist [Mager *et al.*, 1992; Rovnyak, 1995]. The extent of puckering of the 1,4-dihydropyridine "boat" is also affected by C-3 and C-5 substituents (R^3 and R^5), and will be discussed in details in Section 1.4.1.4.

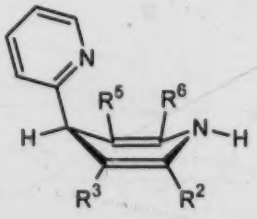


Figure 1.4.1.2d: A 1,4-Dihydropyridine Possessing a Pyridinyl *Anti* Rotameric Orientation

Our research group made a seriess of pyridinyl compounds to study aryl ring torsion [Iqbal *et al.*, 1998]. 4-(2-, 3-, 4-Pyridinyl) dihydropyridines' calcium channel antagonist activites follow the reported potency pattern of ortho ≥ meta >> para. Nuclear Overhauser effect studies were carried out on the C-4 2-pyridinyl compounds. In contrast to the observed aryl *syn* rotamer and aryl *anti* rotamer distribution for most 1,4-dihydropyridines, 2-pyridinyl compounds exist predominantly as the *anti* rotamer in solution at 25 ^{0}C (Figure 1.4.1.2d). Because the pyridinyl nitrogen free electron pair can interact with a nearby proton [Fossheim *et al.*, 1982], it was originally believed that the *anti* compounds are stabilized by an intramolecular hydrogen interaction between the 2-pyridinyl nitrogen and the 1,4-dihydropyridine's N-1 proton [Iqbal *et al.*, 1998]. That intramolecular interaction requires a near perpendicular orientation between the 4-pyridinyl ring and the 1,4-dihydropyridine ring making the "boat" more puckered, and thus the compound is a more potent antagonist [Mager *et al.*, 1992]. In a later X-ray crystallographic study, it was observed that rather than an intramolecular interaction that there was an intermolecular interaction between the pyridinyl nitrogen and the N-1 hydrogen between two dihydropyridine molecules [Parrish *et al.*, 2000].

In regard to the bulkiness of the ortho substituent, in this case, the pyridinyl nitrogen atom is relatively smaller in size to the pyridinyl C-5 carbon and hydrogen atoms, and thus the *anti* rotamer is sterically favorable over the *syn* rotamer.

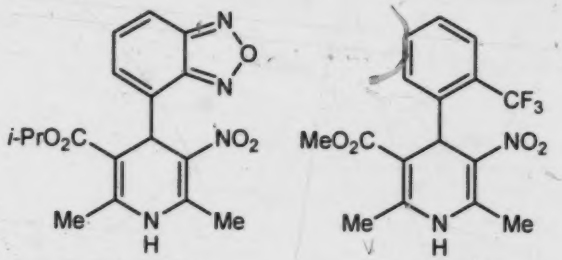
1.4.1.3 The C-4 Chiral Centre

Figure 1.4.1.3a: 1,4-Dihydropyridines with a Chiral Centre at the C-4 Carbon

The C-4 carbon, denoted by an asterisk, is a chiral centre (Figure 1.4.1.3). Because the C-4 aryl group is oriented above the "boat" 1,4-dihydropyridine ring, and the C-4 hydrogen is projected down from the "boat", chirality is determined by the two moieties attached to the C-3 and C-5 carbons rather than the aryl C-4 moiety.

As discussed in Section 1.3.1, each enantiomer can exhibit both calcium channel antagonist and agonist activities. Moreover, the environment surrounding the receptor

affects the type of pharmacological action. In general, under non-extreme conditions, one pharmacological action usually overwhelms its antipode pharmacological action. For example, the two enantiomers of BAY K 8644 show a ~10-fold difference in binding affinity and activity favoring the agonist isomer, whereas the two enantiomers of PN 202 791 show a ~100-fold difference in activities favoring the antagonist enantiomer [Wei *et al.*, 1986] (Figure 1.4.1.3b).



PN 202 791 BAY K 8644

Figure 1.4.1.3b: PN 202 791 and BAY K 8644

In general terms, **the (*S*)-enantiomers are dominantly agonists while the (*R*)-enantiomers are dominantly antagonists** [Mahmoudian & Richards, 1986a; Triggle & Rampe, 1989; Triggle, 1992]. More precisely, for the case of an open-state calcium channel, an (*S*)-enantiomer is more efficient than an (*R*)-enantiomer in maintaining the calcium channel in an open-state. However, in the case of a close-state calcium channel, an (*S*)-enantiomer is no more efficient than an (*R*)-enantiomer in maintaining the calcium channel close-state [Triggle & Rampe, 1989]. In simpler words, an (*S*)-enantiomer has better agonist activity than an (*R*)-enantiomer. However, an (*R*)-enantiomer does not necessarily have better antagonist activity than an (*S*)-enantiomer. In other words, an (*S*)-enantiomer has both agonist and antagonist activity, whereas the (*R*)-enantiomer only has antagonist activity.

1.4.1.4 The C-3 and C-5 Substituents

As mentioned, (*S*)- and (*R*)-configurations are determined by the specific substituent attached to the C-3 and C-5 carbons. When viewed as a molecule docking into a receptor, the IUPAC naming convention for (*S*)- and (*R*)-configuration can lead to erroneous generalizations, such as the statement that "the (*S*)-enantiomers are dominantly agonists while the (*R*)-enantiomers are dominantly antagonists". To resolve

the issue, because the 1,4-dihydropyridine ring is referred to as a "boat", the right side of the "boat" is often referred to as the "starboard side" while the left side is called the "port side" with respect to C-4 as the bow, the axial aryl ring as the bowsprit, and the nitrogen as the stern.

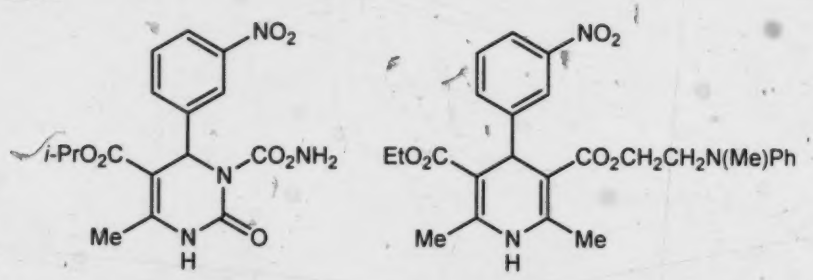


Figure 1.4.1.4a: SQ 32926 and Nicardipine

When the configuration is not known, one side is referred to as the "essential side" while the other is referred to as the "non-essential side". Kappe and Fabian (1997) introduced the concept of "essential" and "non-essential sides" by comparing dihydropyridines and dihydropyrimidines. The 1,2,3,4-tetrahydrodihydropyrimidine, SQ 32926, has been found to be potent relative to related 1,4-dihydropyridine diesters (Figure 1.4.1.4a). SQ 32926's potency suggests that the "non-essential side" of the molecule (the right [starboard] side of the pictogram) is not a strong determinant for antagonist activity as the "essential side" of the molecule (the left [port] side of the pictogram).

An ester substituent at the C-3 or C-5 position on the "essential side" of the molecule provides optimum calcium channel antagonist activity [Bossert & Vater, 1989; Goldmann & Stoltefuss, 1991]. Compounds having ester susbstituents at both C-3 and C-5 exhibit fairly potent antagonist activity [Mager *et al.*, 1992; Triggle, 1992]. Optimum antagonist activity on the "essential side" is reported as **CO_2*i*-Pr > CO_2Me > COMe > CN > H** [Goldmann & Stoltefuss, 1991; Triggle, 1992]. Other non-ester functional groups at C-3 or C-5 position often exhibit very little calcium channel antagonist activity [Bossert & Vater, 1989].

For calcium channel antagonists, X-ray crystallographic studies suggest that the "essential side" ester's carbonyl group is synperiplanar with respect to the dihydropyridine ring double bonds (Figure 1.4.1.4b) [Mahmoudian & Richards, 1986a;

Fossheim, 1987; Kappe, 1997]. Such port side carbonyl oxygen acts as a hydrogen-bond acceptor in binding to a tryptophan residue at the IIIS5/IIIS6 loop portion of the receptor and produces an antagonist response [Natale *et al.*, 1999; Zamponi *et al.*, 2003] (Figure 1.4.1.2b). Thus, in agreement with the potency pattern of CO_2i-Pr > CO_2Me > COMe > CN > H, an electron rich substituent on the essential side of the 1,4-dihydropyridine ring promotes a hydrogen-bonding interaction and improves calcium channel antagonist activity.

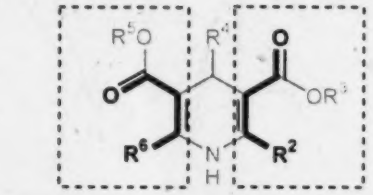


Figure 1.4.1.4b: A 1,4-Dihydropyridine Possessing a Synperiplanar and an Antiperiplanar Ester Substituent

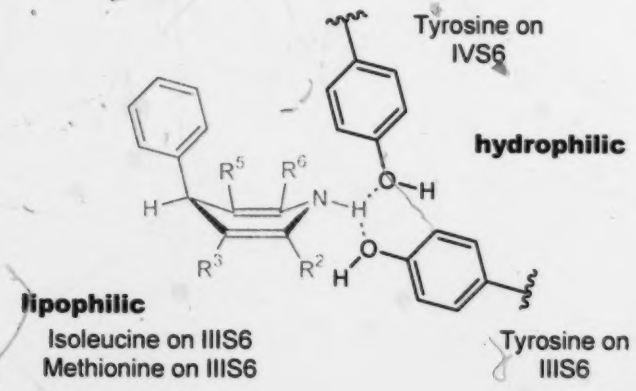


Figure 1.4.1.4c: Second Model – Proposed 1,4-Dihydropyridine Receptor Surface Binding Site

In contrast, Zhorov *et al.* (2001) believe that an electronegative interaction does not play a key role near the C-3 and C-5 regions. Instead, the port side ester undergoes van der Waals interaction in a lipophilic area with an isoleucine and a methionine on the IIIS6 portion of the receptor (Figure 1.4.1.4c). When there is van der Waals interaction, the molecule obstructs the passage of calcium ions through the channel. In the case of

BAY K 8644, the (*S*)-agonist enantiomer has a hydrophilic nitro group on the port side that rolls the molecule away from the IIIS6's hydrophobic region, whereas the (*R*)-antagonist enantiomer has a less hydrophilic methyl ester group on the port side that may interact with the IIIS6's hydrophobic region (Figure 1.4.1.4d). In simpler words, the (*R*)-antagonist enantiomer has van der Waals interaction with the receptor and delays the movement of calcium through the channel, whereas the (*S*)-agonist enantiomer has weaker van der Waals interaction and is oriented in such a way that a gap is created so that calcium can pass through the channel.

Antagonist (R)-(+)-BAY K 8644

Agonist (S)-(-)-BAY K 8644

Adapted from Zhorov *et al.* (2001)

Figure 1.4.1.4d: A Close-Up View of BAY K 8644 Docked in a Pseudo α_1-Subunit of the Calcium Channel Receptor

Small structural modifications of the calcium channel antagonist, nifedipine, at C-3 and/or C-5 gave rise to related compounds such as BAY K 8644, PN 202 791 and CGP 28 392 having calcium channel agonist effects whereby cardiac contractility is enhanced and smooth muscle is contracted (Figure 1.4.1.4e).

Because agonists and antagonists share the same binding site on the calcium channel receptor, the "essential / non-essential side" concept also applies to agonists. For optimum calcium channel agonist activity, the "essential side" has a C-3 **nitro** substituent that is present in BAY K 8644 and PN 202 791 [Triggle, 1992] (Figure 1.4.1.4e). Agonist activity may also predominate in compounds with a fused lactone on the "essential side" such as CGP 28 392 [Rovnyak *et al.*, 1995]. Weaker agonist activity may also be present in compounds with a variety of other functional groups [Goldmann & Stoltefuss, 1991] (Figure 1.4.1.4f).

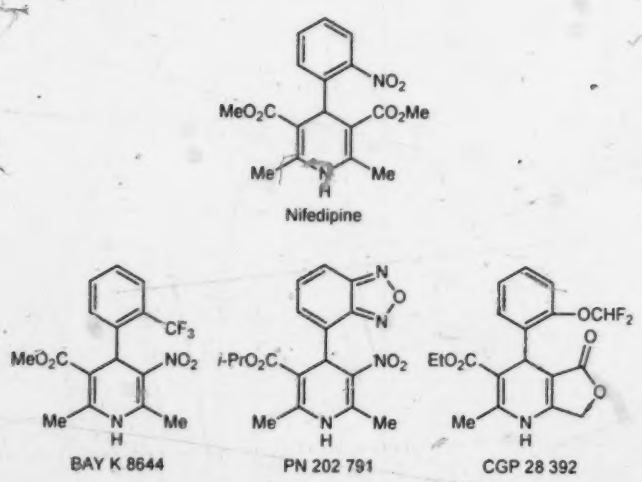


Figure 1.4.1.4e: Nifedipine, BAY K 8644, PN 202 791 and CGP 28 392

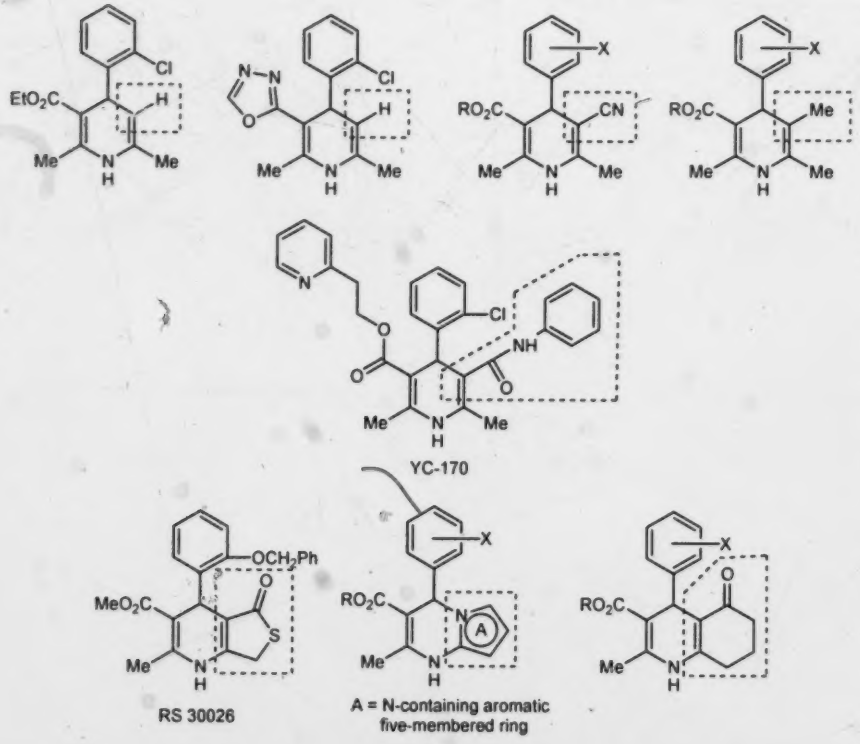


Figure 1.4.1.4f: Weak 1,4-Dihydropyridine Calcium Channel Agonists

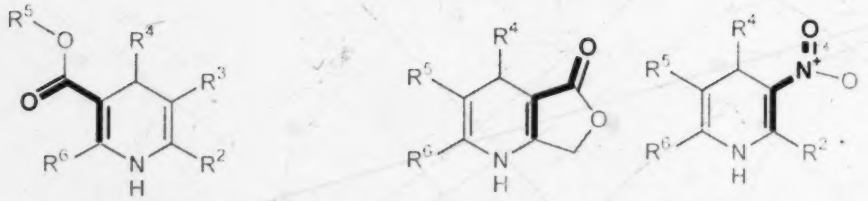


Figure 1.4.1.4g: Antagonist and Agonist 1,4-Dihydropyridines

X-ray crystallographic studies reveal that CGP 28 392 has a lactone carbonyl that is ester antiperiplanar with respect to the dihydropyridine ring double bonds, and the lactone carbonyl oxygen atom acts as a hydrogen-bond acceptor in binding to the receptor that produces the agonist response [Mahmoudian & Richards, 1986a; Fossheim, 1987] (Figure 1.4.1.4g). Using the same argument, in the case of a C-3 nitro substituent, the antiperiplanar oxygen atom of the nitro substituent acts as a hydrogen-bond acceptor in binding to the receptor. Thus, in calcium channel antagonists, a synperiplanar carbonyl oxygen atom of the ester acts as a hydrogen-bond acceptor in binding to the receptor. Whereas in calcium channel agonists, an antiperiplanar carbonyl oxygen atom of the lactone, or the antiperiplanar oxygen atom of the nitro, acts as a hydrogen-bond acceptor in binding to the receptor. In contrast, an X-ray crystallographic study by Langs *et al.* (1991) suggests that instead of the antiperiplanar oxygen atom of the nitro substituent, that it is the synperiplanar oxygen atom of the nitro substituent that is involved in hydrogen-bonding with the receptor.

Calcium channel agonists have a less puckered boat conformation relative to antagonists. Torsion angle constraints of the lactone ring of the agonist, such as CG 28 392, flatten the "boat" and enhance the molecule's agonist effect [Mager *et al.*, 1992; Rovnyak *et al.*, 1995] (Figure 1.4.1.4e). Indeed, X-ray crystallographic studies also suggest that the C-3 nitro substituent of the agonist BAY K 8644 is oriented in the same plane as the dihydropyridine ring [Mahmoudian & Richards, 1986a]. In the case of calcium channel antagonists, the angle between the plane of the 1,4-dihydropyridine ring and the ester group usually ranges from 30 to 60 degrees below the plane, where a larger angle distorts the ring to become more puckered and promote antagonist activity

(Figure 1.4.1.4h). As discussed in Section 1.4.1.2, the extent of flattening of the 1,4-dihydropyridine "boat" is also affected by the C-4 substituent R^4.

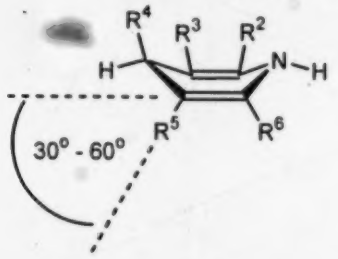


Figure 1.4.1.4h: The C-3 and C-5 Torsion Angle Relative to the Plane of the 1,4-Dihydropyridine Ring

1.4.1.5 The C-2 and C-6 Substituents

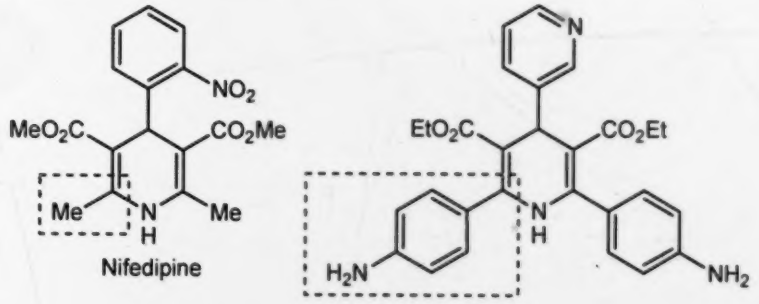


Figure 1.4.1.5a: 1,4-Dihydropyridine Calcium Channel Antagonists Possessing Different "Essential Side" C-2 Substituents

The calcium channel antagonist potency order for substitution on the "essential side" at C-2 (R^2) or C-6 (R^6) position has been reported as **phenyl / aryl ≥ methyl ≥ small alkyl, alkoxy, aminoalkoxy, carbalkoxy > pyridinyl** [Bossert & Vater, 1989; Mager *et al.*, 1992] (Figure 1.4.1.5a).

C-2 or C-6 substitution on the "non-essential side" has improved pharmacodynamic and pharmacokinetic properties with minimal loss to pharmacological activity (Figure 1.4.1.5b). Aminoalkoxy substituents on the "non-essential side", such in the calcium channel antagonist, amlodipine, tend to have a slow onset and long duration of action [Romero *et al.*, 2003]. A slow onset antihypertensive drug is desired in a

maintenance regimen since it minimizes the risk of reflex postural hypotension. Due to the size difference between the R^2 and R^6 substituents, synthesis of the aminoalkyl compounds is much more stereoselective than the neutral 1,4-dihydropyridines [Triggle, 1992]. This remarkable prolonged duration of antihypertensive action is also observed for two other 1,4-dihydropyridines containing a C-2 amino group [Kobayashi *et al.*, 1995a; Kobayashi *et al.*, 1995b]. Moreover, an analogue of nifedipine with a carboxylic acid group at C-2 has improved water solubility while having a moderate coronary vasodilatory effect [Bossert & Vater, 1989].

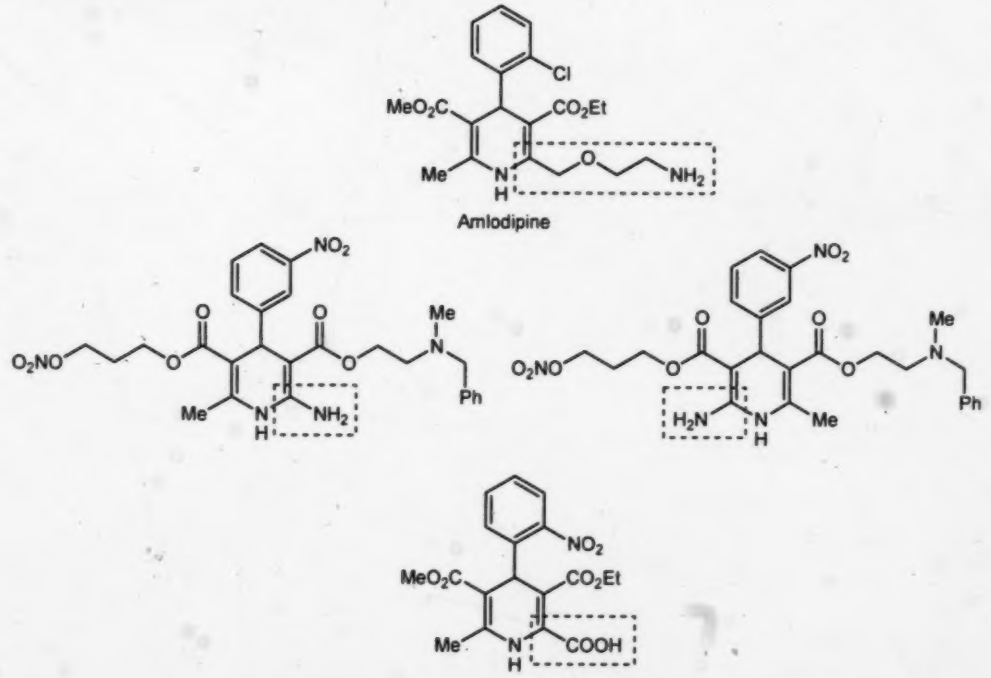
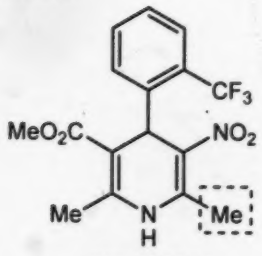


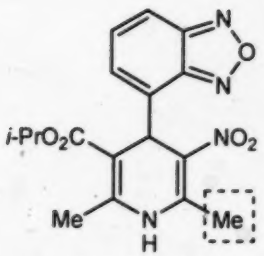
Figure 1.4.1.5b: 1,4-Dihydropyridine Calcium Channel Antagonists Possessing Different "Non-Essential Side" C-2 Substituents

There are considerably fewer examples of 1,4-dihydropyridine agonists compared to 1,4-dihydropyridine antagonists. For calcium channel agonist activity, the order of potency for C-2 substituents on the "essential side" is reported as **methyl ≥ lactone >> lactone isostere** [Bossert & Vater, 1989; Goldmann & Stoltefuss, 1991] (Figure 1.4.1.5c).

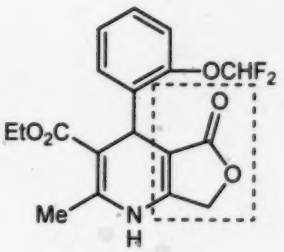
Agonists:



BAY K 8644

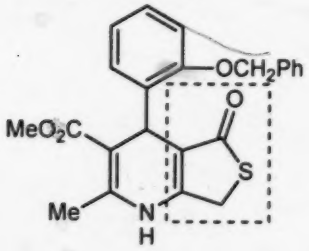


PN 202 791

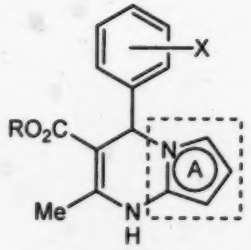


CGP 28 392

Weak Agonists:



RS 30026



A = N-containing aromatic five-membered ring

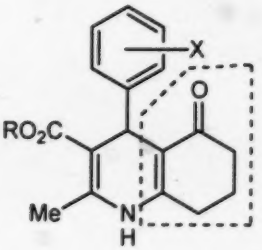


Figure 1.4.1.5c: 1,4-Dihydropyridine Calcium Channel Agonists Possessing Different "Essential Side" C-2 Substituents

1.4.1.6 The N-1 Substituent

The proton attached to the N-1 position greatly contributes to calcium channel modulation activity, because it is most likely involved in a hydrogen-bonding donor interaction with a receptor amino acid [Fossheim *et al.*, 1982; Fossheim, 1987; Langs *et al.*, 1991; Mager *et al.*, 1992; Triggle, 1992; Kappe & Fabian, 1997; Natale *et al.*, 1999; Zhorov *et al.*, 2001; Zamponi *et al.*, 2003]. Indeed, when 1,4-dihydropyridines are metabolized *in vivo*, a Phase I metabolite arising from dehydrogenation of the N-1 hydrogen is an aromatic pyridinyl metabolite [Rämsch *et al.*, 1986]. The pyridinyl metabolite shows no calcium channel modulation activity; most likely because the central planar pyridine ring no longer exists in a "boat" conformation, and the C-4 aryl substituent is no longer pseudoaxial since it is attached to an sp^2 hybridized pyridinyl C-4 atom.

The hydrogen at the N-1 position interacts with a glutamic acid residue on the IIIS6 region of the receptor, and it is critical for calcium channel modulation activity [Fossheim *et al.*, 1982; Fossheim, 1987; Langs *et al.*, 1991; Triggle, 1992; Kappe & Fabian, 1997; Natale *et al.*, 1999; Zamponi *et al.*, 2003] (Figure 1.4.1.2b).

In contrast, Zhorov *et al.* suggest that the N-1 hydrogen interacts with the tyrosine on either the IIIS6 or IVS6 portion of the receptor [Zhorov *et al.*, 2001] (Figure 1.4.1.4c).

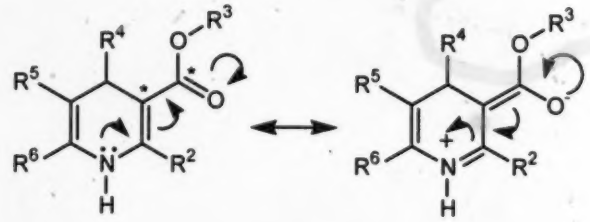


Figure 1.4.1.6a: An Enolamine Electron Delocalization

The N-1 nitrogen is fairly unreactive, possibly due to enolamine electron delocalization [Bossert & Vater, 1989]. X-ray crystallographic studies indicate that the bond length between the two carbons denoted by asterisks is shorter than that of a single bond but longer than that of a double bond, thus suggesting that enolamine electron delocalization does occur [Fossheim, 1987; Langs *et al.*, 1991] (Figure 1.4.1.6a). Moreover, perhaps due to the same reason of enolamine electron delocalization, hydrolysis of the ester at OR^3 has also been found to be somewhat difficult. This electron delocalization occurs on both the "essential and non-essential sides" of the molecule in both agonist and antagonist [Langs *et al.*, 1991]. In the case of an agonist, the delocalization occurs in the direction of the C-3 nitro substituent instead of the C-3 carbonyl substituent.

In general, the presence of an N-1 substituent in the 1,4-dihydropyridine class of compounds reduces activity [Janis & Triggle, 1983]. Various moieties have been attached at the N-1 position which exhibit weaker calcium channel antagonist effects relative to the respective NH-unsubstituted 1,4-dihydropyridines [Bossert & Vater, 1989] (Figure 1.4.1.6b). Our research group has synthesized an N-1 methylated analogue of nifedipine that is unfortunately 17 times less active than nifedipine [Wynn *et al.*, 1988].

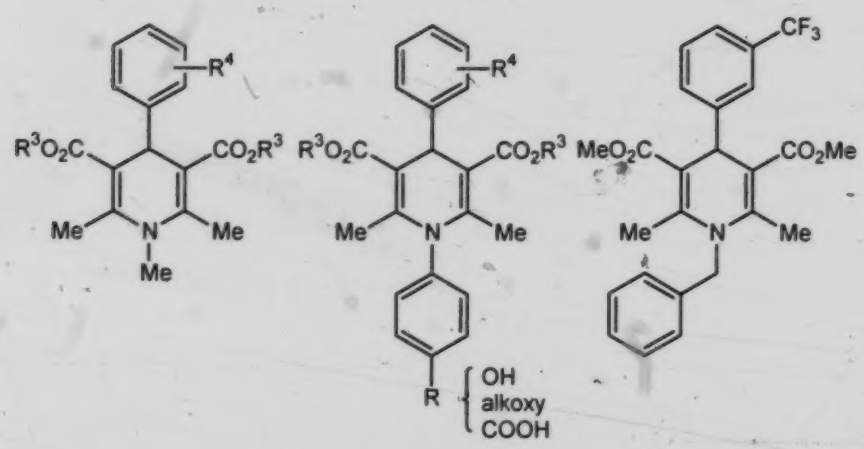


Figure 1.4.1.6b: 1,4-Dihydropyridines Possessing Different N-1 Substituents

1.4.2 The Metabolism of 1,4-Dihydropyridines

In a study by Rämsch *et al.* (1986), nifedipine, nitrendipine and nimotidine were given orally to healthy human volunteers. After a period of time, the volunteers' urine were collected and analyzed for drug metabolites. The study shows that 1,4-dihydropyridines undergo extensive hepatic metabolism and that various metabolites at different biotransformation stages can be identified (Figure 1.4.2). The first main biotransformation step is aromatization of the 1,4-dihydropyridine to the pyridine moiety which abolishes all calcium channel modulation properties. The C-3 and C-5 esters are then hydrolyzed to carboxylic acids. Subsequently, the C-2 and C-6 methyls undergo hydroxylation. If a hydroxycarboxylic acid is formed, intramolecular cyclization occurs, thus forming a lactone moiety. Meyer *et al.* (1983) reported similar results for nimotidine.

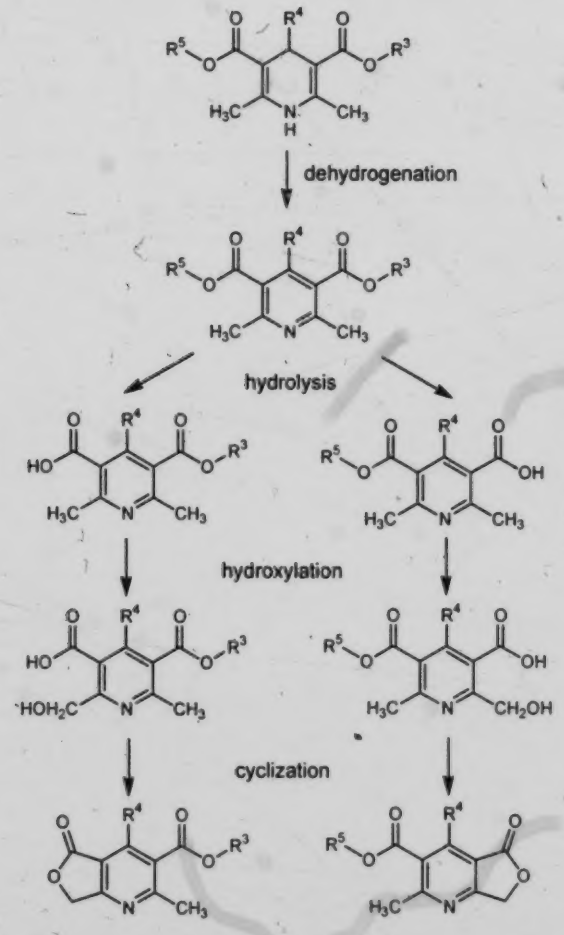
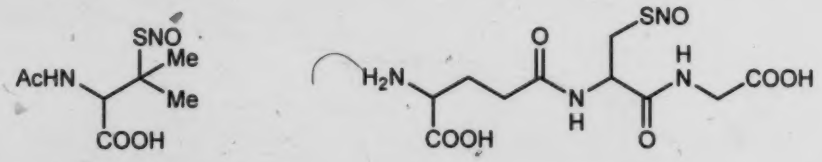


Figure 1.4.2: Metabolites of 1,4-Dihydropyridines

1.5 NITRIC OXIDE DONORS

There are several major classes of nitric oxide donors including nitrosothiols, organic nitrates, organic nitrites, meta-nitric oxide complexes, *N*-nitrosoamines, *N*-hydroxy-*N*-nitrosamines, *N*-nitrosimines, nitrosothiols, *C*-nitroso compounds, diazetine dioxides, furoxans and benzofuroxans, oxatriazole-5-imines, sydnimines, oximes, hydroxylamines, *N*-hydroxyguanidines, hydroxyureas, and diazeniumdiolates. Of the major classes, nitrosothiols, nitrates, furoxans and benzofuroxans, and N^1-substituted diazen-1-ium-1,2-diolates have been investigated as drug hybrids [Gasco *et al.*, 1996; Wang *et al.*, 2002].

1.5.1 Nitrosothiols



S-Nitroso-*N*-acetylpenicillamine *S*-Nitrosoglutathione

Figure 1.5.1a: *S*-Nitroso-*N*-acetylpenicillamine and *S*-Nitrosoglutathione

Nitrosothiols induce cyclic GMP formation, have potent vasodilating effects, and prevent platelet aggregation [Jansen *et al.*, 1992; Mathews & Kerr, 1993; Salas *et al.*, 1994] (Figure 1.5.1a).

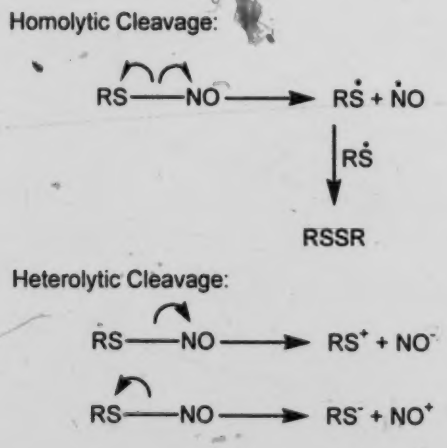


Figure 1.5.1b: The Decomposition of Nitrosothiols

Most nitrosothiols are unstable and will spontaneously release nitric oxide at room temperature upon exposure to air (Figure 1.5.1b). The decomposition of nitrosothiols by both hemolytic and heterolytic cleavage of the S-NO bond is also enhanced by the presence of other thiols, light and metal ions such as Cu^{2+} and Fe^{2+} [Gasco *et al.*, 1996; Wang *et al.*, 2002].

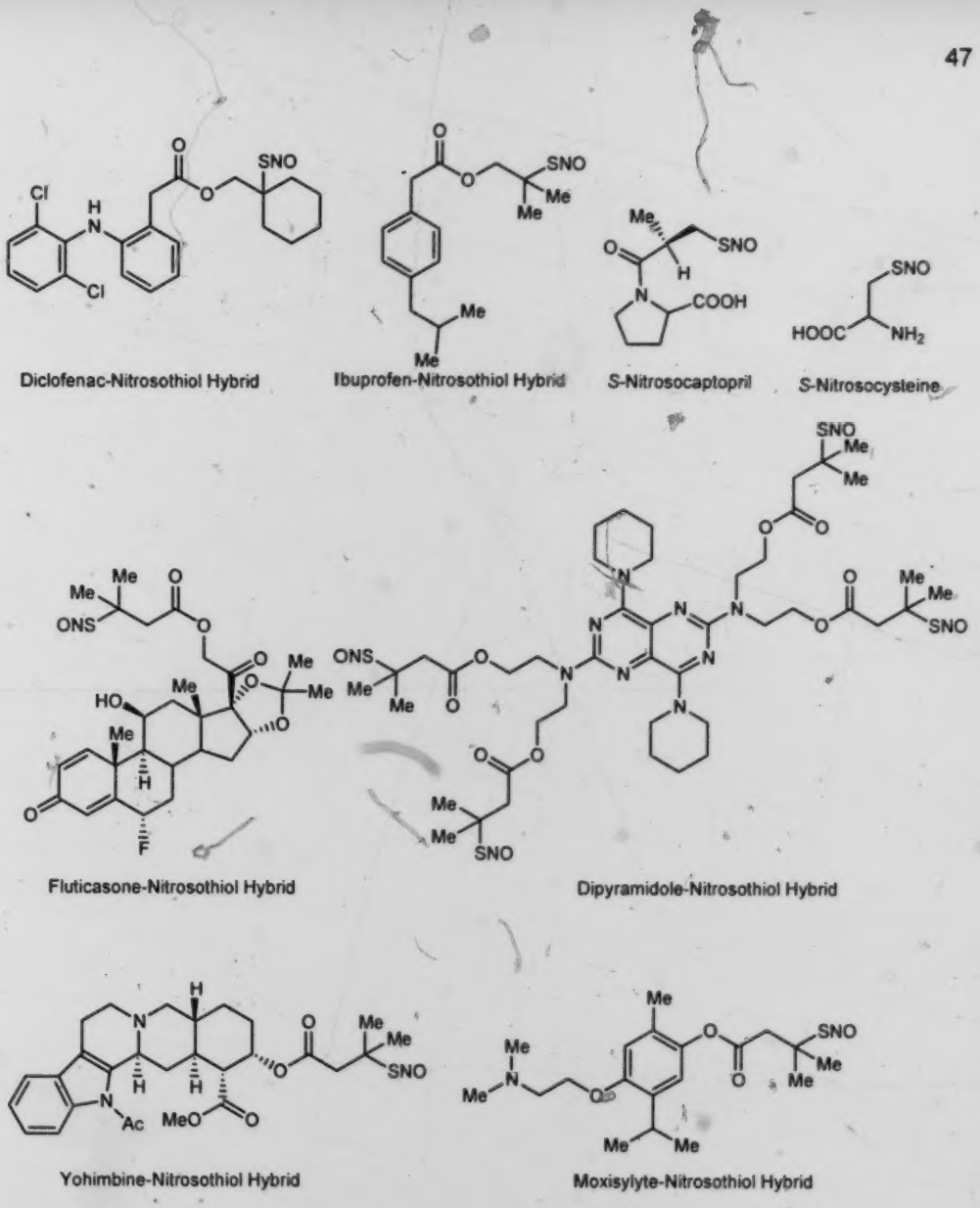
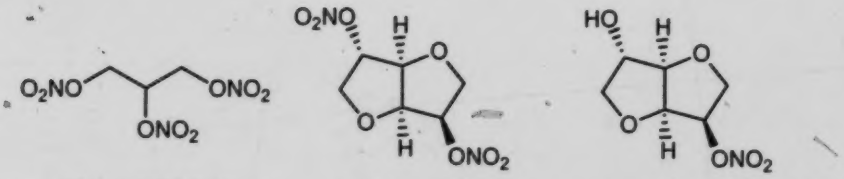


Figure 1.5.1c: Nitrosothiol Hybrids

A nitrosothiol moiety has been attached to the non-steroidal anti-inflammatory agents, diclofenac and ibuprofen (Figure 1.5.1c). A major side-effect of diclofenac and ibuprofen is gastrointestinal ulceration. Nitric oxide protects the mucosal lining of the gastroinstestinal tract by increasing gastric mucosal blood flow [Kerwin & Heller, 1994; Kerwin *et al.*, 1995; Wallace, 1997]. Thus, the diclofenac-nitrosothiol and ibuprofen-nitrosthiol hybrids produce a lower incidence of gastrointestinal side-effects, since the

gastrointestinal healing promoting effect of nitric oxide reduces the prevalence of gastrointestinal lesions [Bandarage *et al.*, 2000]. *S*-Nitrosocaptopril has been studied as a nitric oxide guanylyl cyclase activator and as a captopril vasodilator (an angiotensin converting enzyme type-2 inhibitior) [Loscalzo *et al.*, 1989; Nakae *et al.*, 1995]. *S*-Nitrosocaptopril's vasodilating action behaves more like that of a nitric oxide donor than an angiotensin converting enzyme type-2 inhibitor. The unstable *S*-nitrosocysteine has been studied as an endothelium derived relaxing factor candidate but was unsuccessful [Feelisch *et al.*, 1994; Gasco *et al.*, 1996]. The corticosteroid fluticasone-nitrosothiol and dopamine agonist dipyramidole-nitrosothiol hybrids both exhibit superior pharmacological activity relative to their respective parent compounds [Wang *et al.*, 2002]. When used for local pharmacological treatment of male impotence, the nitrosylate α_1-adrenergic receptor antagonists, yohimbine and moxisylyte, exhibit an increased penile vasodilatory effect over their respective parent compounds in maintaining a sexual erection for an extended period of time [de Tejada *et al.*, 1999].

1.5.2 Organic Nitrates



Glyceryl Trinitrate | Isosorbide Dinitrate | Isosorbide-5-Mononitrate

Figure 1.5.2a: Organic Nitrates

Organic nitrates decrease both the cardiac preload and the afterload by dilating venous and arterial vessels without altering contractility and heart rate [Gasco *et al.*, 1996] (Figure 1.5.2a).

Organic nitrates release nitric oxide upon exposure to thiols and may slowly degrade under light at room temperature (Figure 1.5.2b) [Ferioli, *et al.*, 1995; Wang *et al.*, 2002]. *In vivo*, organic nitrates are metabolized via an NADPH dependent cytochrome P450 pathway, by glutathione-*S*-transferase isozymes, and via reduction by membrane-bound enzyme such as ferrous-heme moieties of hemoglobin and myoglobin, and flavin species (FMN/NADH). Thus, organic nitrates and nitrosothiols may be related

to each other in that organic nitrates may be converted to nitrosothiols by enzyme-activated release of nitric oxide [Yeates, 1992].

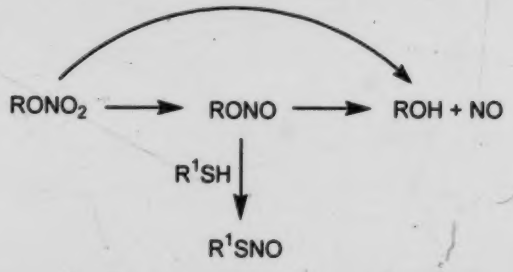


Figure 1.5.2b: The Decomposition of Organic Nitrates

Organic nitrates with neighboring nitrooxy groups such as glyceryl trinitrate (Figure 1.5.2a) can theoretically be hydrolyzed to release three molecules of nitric oxide. However, under physiological conditions, the number of nitrooxy groups present affects the extent of nitric oxide released [Curry & Aburawi, 1985]. Indeed, glyceryl trinitrate readily liberates one mole of nitric oxide with a half-life of 3 to 5 minutes to become glyceryl dinitrate [Noonan *et al.*, 1985]. Glyceryl dinitrates have a half-life of approximately 40 minutes to become glyceryl mononitrates [Noonan & Benet, 1986]. After 24 hour, 22% of the administered dose of glyceryl trinitrate can be collected in the urine as glyceryl mononitrates. Glyceryl dinitrates and glyceryl mononitrates are considered therapeutically inactive [CPhA, 2003]. The reason for the vastly different rate of nitric oxide release is attributed to steric strain differences between the interactions of the nitrooxy groups and the behaviour of enzymatic conversion [Curry & Aburawi, 1985; Fung, 1992; Seth & Fung, 1993].

Acetyl salicylic acid coupled to a nitrooxy donor moiety, such as NCX-4016, NCX-4215 and acetylsalicylate nitroxybutyl ester, have been shown to possess anti-aggregating effects for platelets due to the inhibitory effect of acetyl salicylic acid on the cyclooxygenase-1 isozyme, and the induced formation of soluble guanylyl cyclase by nitric oxide (Figure 1.5.2c) [Wallace & Cirino, 1994; Lechi, *et al.*, 1996; del Soldato *et al.*, 1999; Fiorucci *et al.*, 2000a; Momi *et al.*, 2000; Napoli *et al.*, 2001]. Moreover, NCX-4016 retains the anti-thrombotic and anti-inflammatory effects attributed to cyclooxygenase inhibition, while providing nitric oxide's protection against gastric mucosa ulceration [Takeuchi *et al.*, 1998; Wallace *et al.*, 1999a; Al-Swayeh *et al.*, 2000; Fiorucci, *et al.*, 2000b]. Salicylate derivatives with a coupled isosorbide mononitrate or

nitrooxy moiety have also been synthesized [Wang *et al.*, 2002]. Other non-steroidal anti-inflammatory agents coupled to a nitrooxy moiety include mesalamine nitrooxybutyl ester, flurbiprofen nitrooxybutyl ester, nitrofenac (an analogue of diclofenac) and ketoprofen nitrooxybutyl ester [Wallace *et al.*, 1994; Minuz *et al.*, 1995; Wallace *et al.*, 1999b].

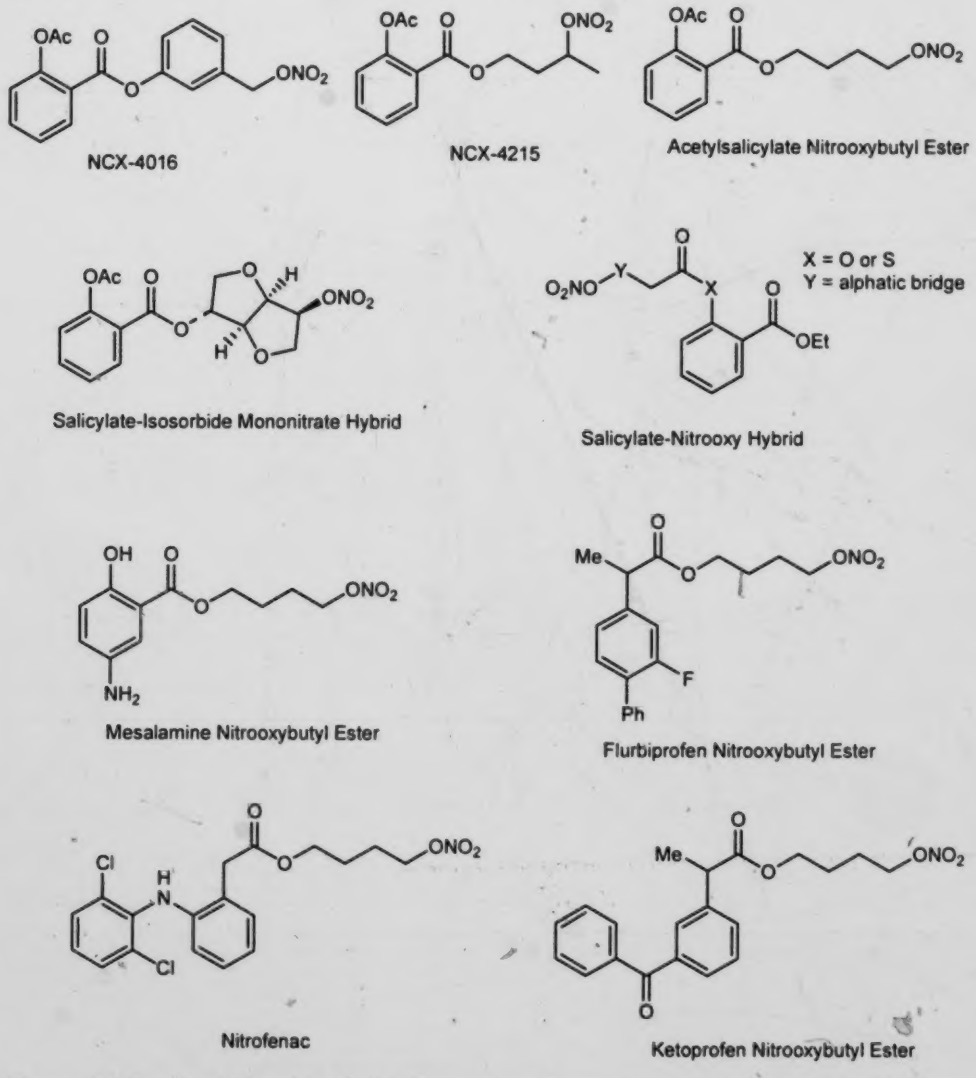


Figure 1.5.2c: Non-Steroidal Anti-Inflammatory Agents Possessing a Nitrooxy Moiety

Nitrooxy hybrids of the steroidal anti-inflammatory agent ursodeoxycholic acid (NCX-1000) and prednisolone (NCX-1015) exhibit enhanced anti-inflammatory and

immunosuppressive effects [Paul-Clark *et al.*, 2000; Fiorucci *et al.*, 2001] (Figure 1.5.2d). Moreover, NCX-1000 also has anti-apoptotic activity.

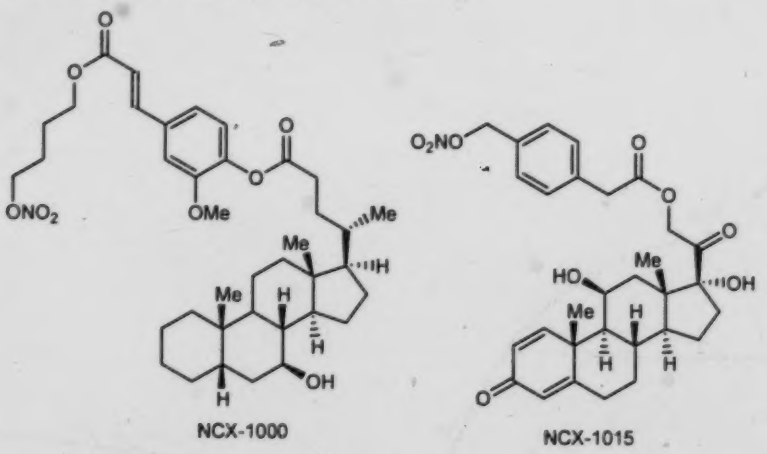


Figure 1.5.2d: Steroidal Anti-Inflammatory Agents Possessing a Nitrooxy Moiety

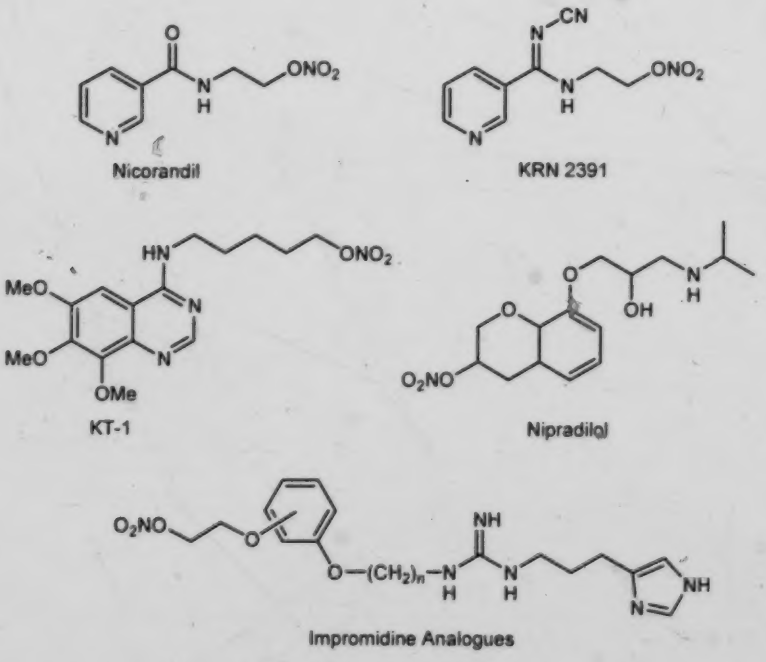


Figure 1.5.2e: NCX-4016, Nicorandil, KRN 2391, KT-1, Nipradilol, and Analogues of Impromidine

Nicorandil and KRN 2391 are potassium channel agonists whose potency is enhanced by the pharmacological properties of organic nitrates [Edwards & Weston, 1990; Evans & Taylor, 1994] (Figure 1.5.2e). KT-1 and nipradilol induce mixed organic nitrate and β-adrenergic antagonistic activities [Uchida *et al.*, 1983; Ohira *et al.*, 1985; Miyamoto *et al.*, 1991]. A group of histamine type-2 agonists with a nitrooxy moiety has been synthesized based on impromidine as a site-targeting carrier [Leurs *et al.*, 1995].

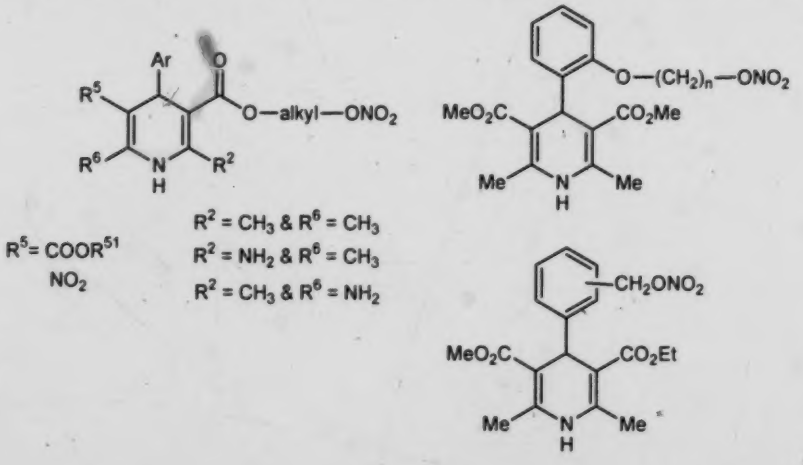


Figure 1.5.2f: 1,4-Dihydropyridine Calcium Channel Modulators Possessing a Nitrooxy Moiety

Various 1,4-dihydropyridine calcium channel modulators coupled to a nitrooxy moiety have been synthesized by our and other research groups [Kawashima *et al.*, 1993; Miyata *et al.*, 1993; Ogawa *et al.*, 1993a; Ogawa *et al.*, 1993b; Kobayashi *et al.*, 1995a; Kobayashi *et al.*, 1995b; Iqbal & Knaus, 1996; Lehmann *et al.*, 1997; Shan & Knaus, 1999; Miri *et al.*, 2000] (Figure 1.5.2.f).

1.5.3 Furoxans and Benzofuroxans

Furoxans and benzofuroxans represent another class of nitric oxide donor. Furoxans exert various nitric oxide-derived pharmacological activities such as cytotoxicity, mutagenicity, immunosuppression, central muscle relaxant properties, anticonvulsive effects, monoamine oxidase inhibition, and direct vasodilator and blood pressure lowering activities [Wang *et al.*, 2002]. Benzofuroxans are potent antileukemic and immunosuppressive drugs that have been shown to inhibit *in vitro* RNA synthesis in

sheep lymphocytes [Ghosh, 1968; Ghosh & Whitehouse, 1968; Ghosh & Whitehouse, 1969]. Derivatives of benzofuroxan including furazanobenzofuroxan, furoxanobenzofuroxan and furoxanobenzothiadiazole are potent *in vitro* and *in vivo* vasodilators [Ghosh & Everitt, 1977; Medana *et al.*, 1999]. Indeed, some furoxans and benzofuroxans increase the level of cellular cyclic GMP in human platelets thereby preventing platelet aggregation, and activate soluble guanylyl cyclase in the presence of thiols while being tolerance-resistant nitrovasodilators [Feelisch *et al.*, 1992; Ghigo *et al.*, 1992; Wang *et al.*, 2002]. Moreover, some furoxans and benzofuroxans can also be used as herbicidal agents against *Triticum aestium* (winter wheat) [Cerecetto *et al.*, 1999].

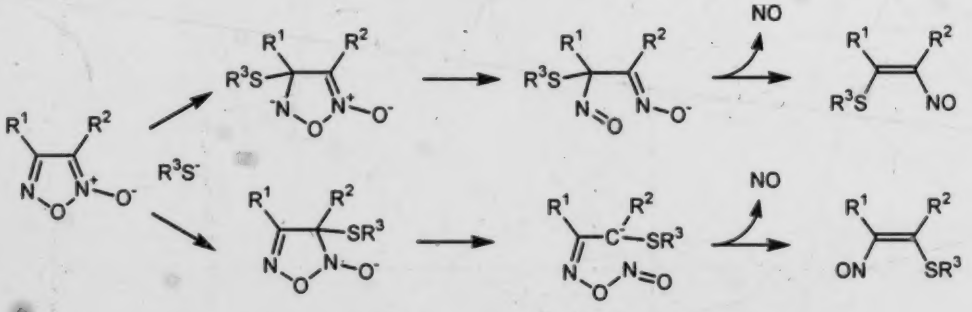


Figure 1.5.3a: The Decomposition of Furoxans and Benzofuroxans

Furoxans and benzofuroxans, much like organic nitrates, release nitric oxide when exposed to thiols [Feelisch *et al.*, 1992; Civelli *et al.*, 1994; Medana *et al.*, 1994; Ferioli, *et al.*, 1995; Civelli *et al.*, 1996; Medana *et al.*, 1999] (Figure 1.5.3a).

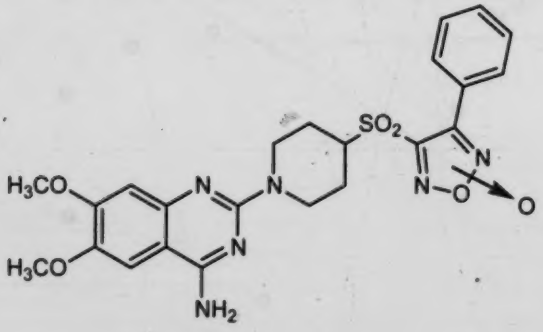


Figure 1.5.3b: Prazosin-Furoxan Hybrids

Hybrid molecules coupling the antihypertensive drug prazosin and a furoxan moiety have been synthesized. Results acquired using these compounds demonstrated that the relative α_1-adrenergic receptor antagonistic vasodilation and nitric oxide-vasodilation effects can be altered by modifying a portion of the molecule. The furoxansulfonyl piperidine derivatives exert an appropriate balance between the α_1-antagonistic vasodilation effect and nitric oxide-mediated vasodilation effect (Figure 1.5.3b) [Fruttero *et al.*, 1995].

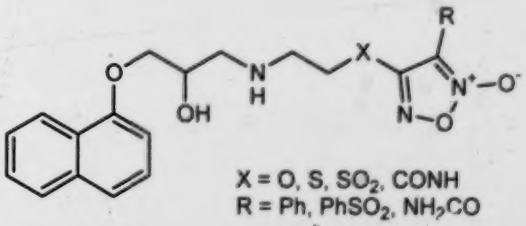


Figure 1.5.3c: β_2-Antagonist-Furoxan Hybrids

Hybrids with mixed nitric oxide-mediated vasodilation and β_2-adrenergic receptor antagonist vasodilation have also been reported (Figure 1.5.3c). Much like the prazosin hybrids, the balance of pharmacological effects between nitric oxide-dependent vasodilation and β_2-antagonist properties is dependent upon the specific attached moieties [Boschi *et al.*, 1997].

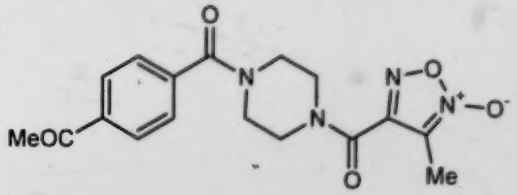


Figure 1.5.3d: Nicorandil-Furoxan Hybrid

A group of nicorandil derived furoxanyl compounds possessing both cardiovascular and cerebrovascular antihypertensive activities has been synthesized. The nicorandil-furoxan hybrid that is illustrated by Figure 1.5.3d, exhibited a gradual and sustained antihypertensive effect in anaesthetized rats [Mu *et al.*, 2000].

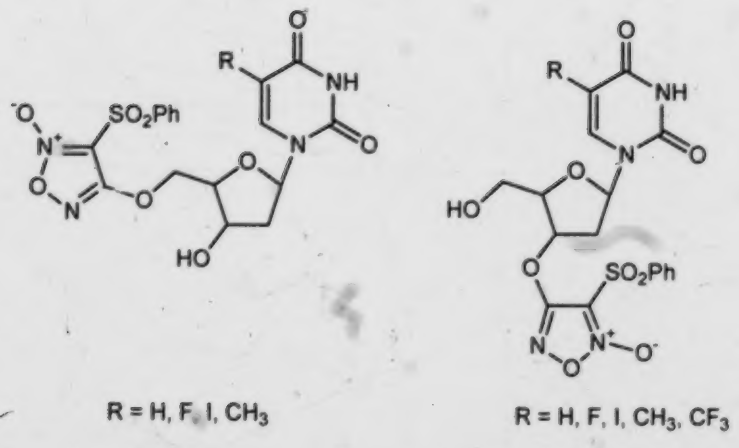
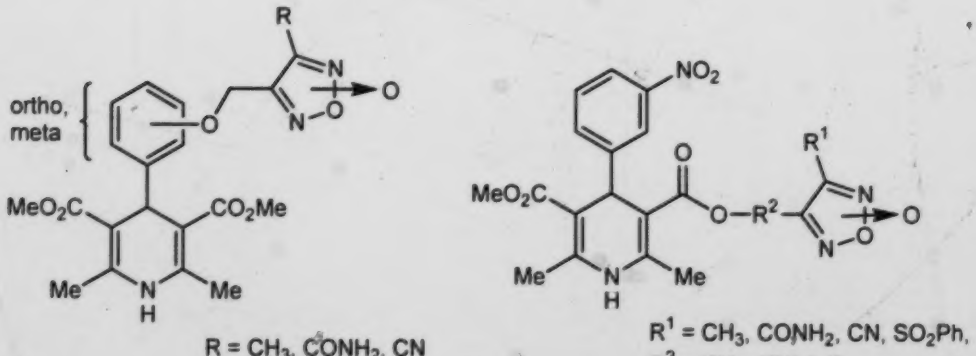


Figure 1.5.3e: Hybrid Furoxan-Nucleoside Anticancer Agents



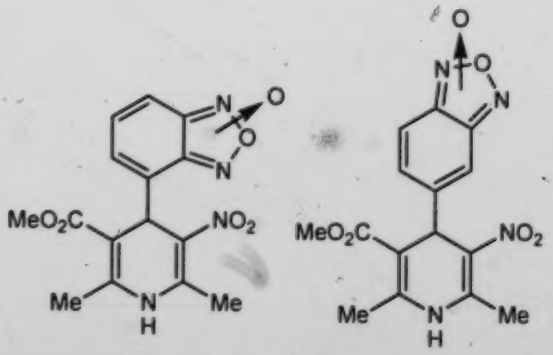


Figure 1.5.3f: 1,4-Dihydropyridine Calcium Channel Modulators with a Furoxan or Benzofuroxan Moiety

Our research group has attached furoxan moieties to nucleosides as potential hybrid nitric oxide donor-nucleoside anticancer agents (Figure 1.5.3e). The resulting compounds were extremely cytotoxic to both normal and cancer cell lines. This data suggest that the *in vivo* toxicity of furoxan nitric oxide donor compounds toward normal cell lines may represent an obstacle to their potential clinical use [Moharram *et al.*, 2004].

1,4-Dihydropyridine calcium channel antagonists conjugated furoxanyl moieties possess both nitric oxide-dependent and calcium channel antagonist vasodilator activities [Di Stilo *et al.*, 1998; Cena *et al.*, 2001] (Figure 1.5.3f). 1,4-dihydropyridine calcium channel agonists possessing a benzofuroxanyl moiety have also been synthesized [Visentin *et al.*, 1999].

1.5.4 N^1-Substituted Diazen-1-ium-1,2-diolates

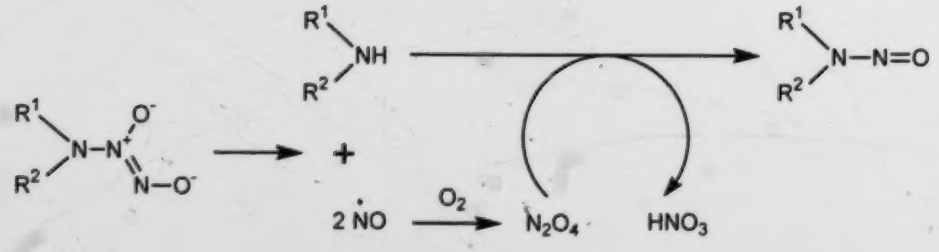


Figure 1.5.4a: The Decomposition of N^1-Substituted Diazen-1-ium-1,2-diolates

N^1-Substituted diazen-1-ium-1,2-diolates possess strong vasodilating and anitproliferative activities [Diodati *et al.*, 1993; Morley *et al.*, 1993; Saavedra *et al.*, 2000].

N^1-Substituted diazen-1-ium-1,2-diolates do not require thiols to release nitric oxide (Figure 1.5.4a) [Keefer, 1998]. Although two molecules of nitric oxide are produced, the amine product may recapture one molecule of nitric oxide. The decomposition of nitric oxide to dinitrogen tetroxide will be discussed further in Section 1.5.6. Nitric oxide release is enhanced by acid and retarded by base when exposed to air [Hrabie & Keefer, 2002]. In physiological solutions, N^1-substituted diazen-1-ium-1,2-diolates spontaneously release nitric oxide with a rate that is dependent upon the nature of the secondary amino moiety [$R^1(R^2)N$] with a half-life ranging from 3 seconds to 56 hours [Hrabie *et al.*, 1993; Keefer, 1998].

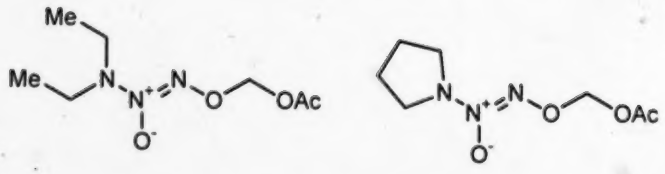
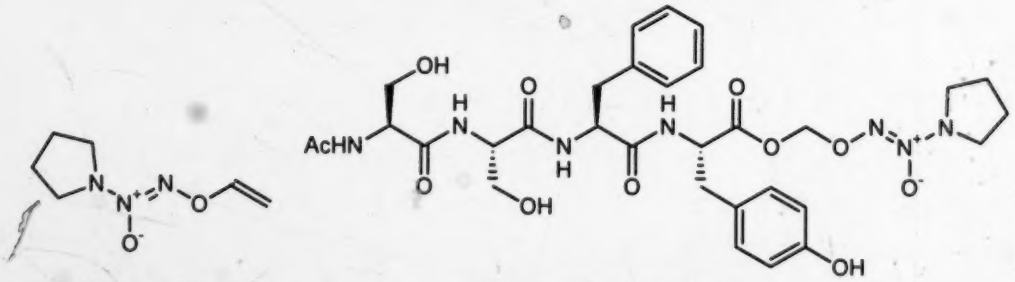


Figure 1.5.4b: O^2-Acetoxymethyl 1-(*N*-Substituted)diazen-1-ium-1,2-diolates

To prevent the immediate release of nitric oxide, an O^2-sodium or potassium salt of 1-(*N*-substituted)diazen-1-ium-1,2-diolate can be converted to a biolabile O^2-acetoxymethyl 1-(*N*-substituted)diazen-1-ium-1,2-diolate derivative that is stable in neutral aqueous media, but which releases nitric oxide upon metabolism by esterases. O^2-acetoxymethyl derivatives are often the easiest to hydrolyze while remaining somewhat stable under neutral aqueous media [Saavedra *et al.*, 2000] (Figure 1.5.4b).



Liver-Selective Targeting

Prostate-Specific Antigen Substrate

Figure 1.5.4c: Organ-Selective Targeting N^1-Substituted Diazen-1-ium-1,2-diolates

Nitric oxide releasing N^1-substituted diazen-1-ium-1,2-diolate moieties have been designed to offer substantial cell-targeting advantages to nitric oxide-sensitive human leukemia cells. Results indicate a two-fold increased in leukemia cell anti-proliferative potency when the diazeniumdiolate moiety is attached. Moreover, organ-selective targeting can also be obtained by modifying the protection group (Figure 1.5.4c) [Saavedra *et al.*, 1997; Tang *et al.*, 2001].

A group of O^2-(glycoside) 1-(*N*-substituted)diazen-1-ium-1,2-diolate hybrids has been synthesized to improve stability as compared to the parent 1-(*N*-substituted)diazen-1-ium-1,2-diolate salts [Wu *et al.*, 2001] (Figure 1.5.4d). These glycoside hybrids can readily release nitric oxide upon cleavage by their corresponding glycosidases.

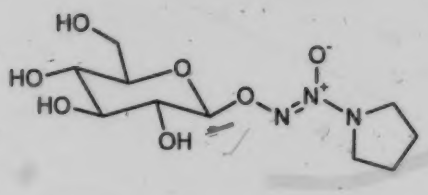


Figure 1.5.4d: A Glycoside Possessing an N^1-Substituted Diazen-1-ium-1,2-diolate Moiety

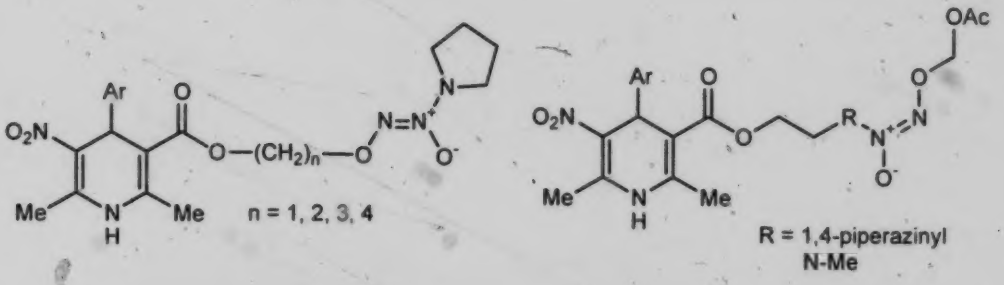


Figure 1.5.4e: 1,4-Dihydropyridine Calcium Channel Agonists Possessing an N^1-Substituted Diazen-1-ium-1,2-diolate Moiety

Our research group has attached a N^1-substituted diazen-1-ium-1,2-diolate moiety to the C-5 position on the "non-essential" side of 1,4-dihydropyridine calcium channel agonists [Velázques *et al.*, 2003; Velázquez & Knaus, 2004] (Figure 1.5.4e).

1.5.5 BAY K 8644

Interestingly, nitro-containing compounds such as the 1,4-dihydropyridine calcium channel agonist, BAY K 8644, release nitric oxide when exposed to light [Bauer & Fung, 1994] (Figure 1.4.1.3b). The extent of nitric oxide produced depends upon the intensity of the irradiated light.

1.5.6 The Decomposition of Nitric Oxide

In 1992, nitric oxide was named molecule of the year by the journal *Science*. In 1998, the importance of nitric oxide was underscored by the award of the Nobel Prize in Medicine to three outstanding cardiovascular investigators, namely Robert Furchgott,

Louis Ignarro and Ferid Murad [Jugdutt, 2002]. In fact, since 1997, nitric oxide even has a bi-/tri-monthly self-devoted journal appropriately entitled, *Nitric Oxide*.

Nitric oxide is an uncharged, paramagnetic molecule containing an unpaired electron [Kerwin *et al.*, 1995]. Nitric oxide in the pure state under standard temperature and pressure exists as a gas. However, in a physiological environment, with the exception of the lung, nitric oxide exists as a dissolved nonelectrolyte. Thus, under virtually all biologically relevant conditions, nitric oxide does not behave as a gas. In the case of nitric oxide donors, the released nitric oxide behaves as a dissolved molecule. Indeed, nitric oxide is moderately soluble in water with a concentration in a saturated solution of 1.9 mM (25^0C), and it tends to dissolve selectively in the membrane and lipid phases of cells [Shaw & Vosper, 1977]. Consequently, dissolved nitric oxide has a much longer half-life than the *in vitro* 5-10 seconds half-life of its gaseous counterpart [Ignarro, 1990]. The rate of decomposition of dissolved nitric oxide depends on the nature of the environment in which nitric oxide is situated. Accordingly, dissolved nitric oxide is relatively stable at 100 nM with a half-life of 80 minutes, but it is rapidly inactivated at a higher concentration of 10 μM with a half-life of 50 seconds [Schmidt *et al.*, 1997].

$$2\,\dot{N}O + O_2 \longrightarrow 2\,\dot{N}O_2$$

$$[NO^+] + OH^- \longrightarrow NO_2^- + H^+$$

$$\dot{N}O_2 + \dot{N}O \longrightarrow N_2O_3$$

$$[NO^+][NO_2^-] + H_2O \longrightarrow 2\,NO_2^- + 2\,H^+$$

$$\dot{N}O_2 + \dot{N}O_2 \longrightarrow N_2O_4$$

$$[NO^+][NO_3^-] + H_2O \longrightarrow NO_2^- + NO_3^- + 2\,H^+$$

Figure 1.5.6: The Decomposition of Nitric Oxide

The decomposition of nitric oxide has been extensively studied [Ridd, 1961; Wink *et al.*, 1993; Schmidt *et al.*, 1997] (Figure 1.5.6). Two molecules of nitric oxide react with one molecule of oxygen to produce two molecules of paramagnetic radical, nitrogen dioxide (NO_2). Nitrogen dioxide can react further with either another molecule of nitric oxide to produce dinitrogen trioxide (N_2O_3), or with another molecule of nitrogen dioxide to produce dinitrogen tetroxide (N_2O_4). In aqueous solution, dinitrogen trioxide and

dinitrogen tetroxide dissociate into $[NO^+][NO_2^-]$ and $[NO^+][NO_3^-]$ respectively, thus a nitrosonium ion ($[NO^+]$) is produced per molecule. In water, nitrosonium ion transfers to hydroxyl and yields depending on the pH of the aqueous medium either nitrous acid (HNO_2) or nitrite (NO_2^-). By the same line of reasoning, dinitrogen trioxide reacts with water to yield either nitrous acid or nitrite. Dinitrogen tetroxide reacts with water to yield nitrite (NO_2^-) and nitrate (NO_3^-), or their respective conjugate acids, nitrous acid (HNO_2) and nitric acid (HNO_3).

2 RESEARCH OBJECTIVES

1,4-Dihydropyridine calcium channel *agonists* is a class of positive inotropes that increases cardiac contractile force without increasing heart rate. However, the calcium channel agonists' adverse vasocontrictive action makes them unsuitable for the treatment of heart failure [Erhardt, 1987; Goldenberg & Cohn, 1987; Holland *et al.*, 1989]. We hypothesize that the addition of a nitric oxide donor moiety to a 1,4-dihydropyridine calcium channel agonist can counter-balance the vascular constriction side-effect.

1,4-Dihydropyridine calcium channel *antagonists* are used as antihypertensive agents [CPhA, 2003], and may also be useful in treating heart failure. Unfortunately, the first generation 1,4-dihydropyridine calcium channel antagonist, nifedipine, has a negative inotropic effect and induces reflex neurohumoral activation [Ooi & Colucci, 2001]; while the second generation 1,4-dihydropyridines, amlodipine and felodipine, showed no improvement in exercise capacity, or quality of life, or survival [Packer *et al.*, 1996; Cohn *et al.*, 1997]. We hypothesize that the addition of a nitric oxide donor moiety to a 1,4-dihydropyridine calcium channel antagonist can enhance the vasodilatory effect, while alleviating the undesired pharmacological effects.

Of the major classes of nitric oxide donors, nitrosothiols, organic nitrates, furoxans and benzofuroxans, and N^1-substituted diazen-1-ium-1,2-diolates have been investigated as drug hybrids [Gasco *et al.*, 1996; Wang *et al.*, 2002]. Nitrosothiol-dihydropyridine hybrids are not suitable candidates for our research, because nitrosothiols are known to be highly unstable [Gasco *et al.*, 1996; Wang *et al.*, 2002].

First Project:

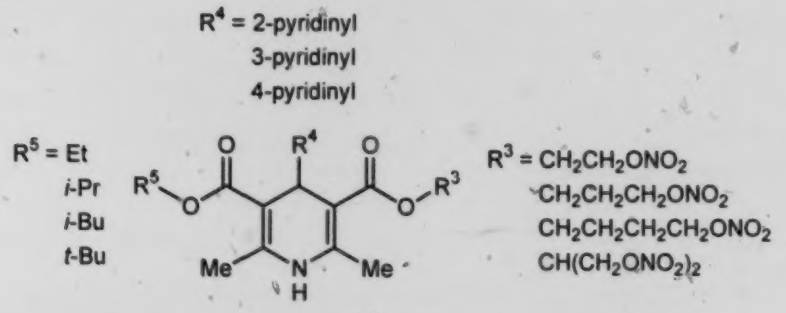


Figure 2a: 1,4-Dihydropyridine Calcium Channel Antagonists Possessing a C-3 or C-5 Substituted Nitrooxy Moiety

We are interested in studying the structure-activity relationships of 1,4-dihydropyridine calcium channel *antagonists* containing a nitrooxy moiety as a class of organic nitrate hybrids (Figure 2a) (Section 3.1.1).

Second Project:

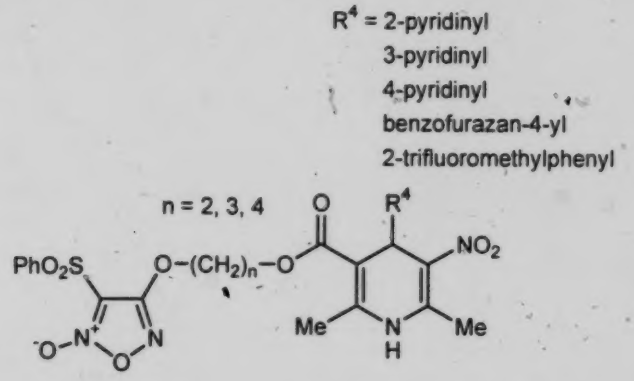


Figure 2b: 1,4-Dihydropyridine Calcium Channel Agonists Possessing a C-5 Substituted Furoxanyl Moiety

We are interested in studying the structure-activity relationships of 1,4-dihydro-3-nitropyridine calcium channel *agonists* containing a furoxanyl moiety (Figure 2b) (Section 3.1.2).

Third Project:

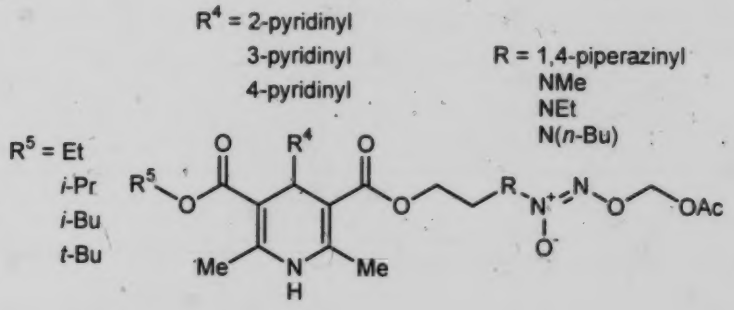


Figure 2c: 1,4-Dihydropyridine Calcium Channel Antagonists Possessing a C-3 or C-5 Substituted O^2-acetoxymethyl-1-(*N*-ethyl-*N*-alkylamino, or 4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate Moiety

We are interested in studying the structure-activity relationships of 1,4-dihydropyridine calcium channel *antagonists* containing an N^1-substituted diazen-1-ium-1,2-diolate (Figure 2c). Our research group has recently published work on 1,4-dihydro-3-nitropyridine calcium channel *agonists* containing an N^1-substituted diazen-1-ium-1,2-diolate [Velázquez and Knaus, 2004] (Sections 3.1.3 and 3.1.4).

3 MATERIALS AND METHODS

3.1 CHEMISTRY

Melting points were determined using a Thomas-Hoover capillary apparatus and are uncorrected. Melting points greater than 300°C could not be reliably determined from the Thomas-Hoover capillary apparatus. Infrared (IR) spectra were recorded using a Nicolet 550 Groups II Magna FT-IR spectrometer. Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a Bruker AM-300 spectrometer and chemical shifts are expressed in parts per million (ppm, δ) relative to tetramethylsilane as internal standard. Spin multiplets are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet) and m (multiplet). Coupling constants (*J*) are given in Hertz (Hz). ^{13}C NMR spectra were acquired using the *J* modulated spin echo technique where methyl and methane carbons appear as positive deflections, and methylene and quarternary carbon resonances appear as negative deflections. The assignment of exchangeable protons (N*H*, O*H*) was confirmed by the addition of D_2O. Ultraviolet spectra and quantitative analyses were measured using a Phillips PU 8740 UV/VIS scanning spectrophotometer and a Thermo Labsystem Multiscan Ascent. Quantitative structure-activity correlations were determined on a Windows personal computer using the Alchemy 2000 (Ver. 2), and Microsoft Excel (Ver. 2003). Microanalyses were performed for C, H, N and Cl (Micro Analytical Service Laboratory, Department of Chemistry, University of Alberta), and water of hydration was not verified analytically. Nominal mass, positive polarity, electrospray spectra were acquired using a Kratos MS-50 (for compounds **46-48**, and **56b**) and Waters Micromass ZQ mass spectrometer (for compounds **42a** and **58-65**). Preparative thin layer chromatography (PTLC) was performed using Macherey Nagel P/UV254 plates, 2.0 mm in thickness. Silica gel column chromatography was performed using Silicycle silica gel (70-230 mesh). Glyceryl trinitrate [Marken *et al.*, 1977], benzofurazan-4-carboxaldehyde (**4e**) [Heitzmann, 1987], isobutyl and *t*-butyl 3-aminocrotonates (**5c-d**) [Koo, 1953], 3,4-bis(benzenesulfonyl)furoxan (**18**) [Kelly *et al.*, 1977], nitroacetone (**20**) [Hurd & Nilson, 1955], 2-cyanoethyl 3-aminocrotonate (**21**) [Ogawa *et al.*, 1993a], chloromethyl acetate (**49**) [Tendler *et al.*, 1987], O^2-acetoxymethyl 1-(4-[2-hydroxyethyl]piperazin-1-yl)diazen-1-ium-1,2,-diolate (**54a**) and O^2-acetoxymethyl 1-(*N*-[2-hydroxyethyl]-*N*-methylamino)diazen-1-ium-1,2,-diolate (**54b**) [Velázquez & Knaus, 2004] were prepared

according to literature procedures. All other reagents were purchased from Aldrich Chemical (Milawukee, WI).

3.1.1 1,4-Dihydropyridine-Organic Nitrate Hybrids

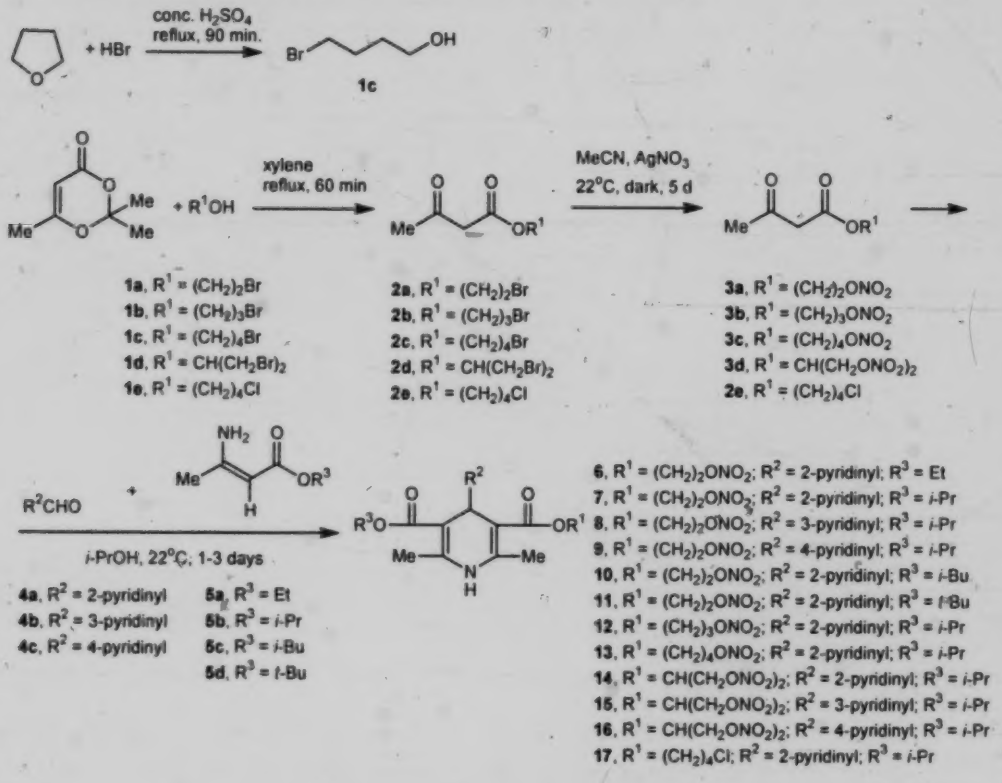

conc. H_2SO_4
reflux, 90 min.
+ HBr
Br
OH
1c
+ R^1OH
xylene
reflux, 60 min
MeCN, $AgNO_3$
22°C, dark, 5 d
1a, $R^1 = (CH_2)_2Br$
1b, $R^1 = (CH_2)_3Br$
1c, $R^1 = (CH_2)_4Br$
1d, $R^1 = CH(CH_2Br)_2$
1e, $R^1 = (CH_2)_4Cl$
2a, $R^1 = (CH_2)_2Br$
2b, $R^1 = (CH_2)_3Br$
2c, $R^1 = (CH_2)_4Br$
2d, $R^1 = CH(CH_2Br)_2$
2e, $R^1 = (CH_2)_4Cl$
3a, $R^1 = (CH_2)_2ONO_2$
3b, $R^1 = (CH_2)_3ONO_2$
3c, $R^1 = (CH_2)_4ONO_2$
3d, $R^1 = CH(CH_2ONO_2)_2$
3e, $R^1 = (CH_2)_4Cl$
R^2CHO
i-PrOH, 22°C, 1-3 days
4a, R^2 = 2-pyridinyl
4b, R^2 = 3-pyridinyl
4c, R^2 = 4-pyridinyl
5a, R^3 = Et
5b, R^3 = i-Pr
5c, R^3 = i-Bu
5d, R^3 = t-Bu
6, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = Et
7, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = i-Pr
8, $R^1 = (CH_2)_2ONO_2$; R^2 = 3-pyridinyl; R^3 = i-Pr
9, $R^1 = (CH_2)_2ONO_2$; R^2 = 4-pyridinyl; R^3 = i-Pr
10, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = i-Bu
11, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = t-Bu
12, $R^1 = (CH_2)_3ONO_2$; R^2 = 2-pyridinyl; R^3 = i-Pr
13, $R^1 = (CH_2)_4ONO_2$; R^2 = 2-pyridinyl; R^3 = i-Pr
14, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 2-pyridinyl; R^3 = i-Pr
15, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 3-pyridinyl; R^3 = i-Pr
16, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 4-pyridinyl; R^3 = i-Pr
17, $R^1 = (CH_2)_4Cl$; R^2 = 2-pyridinyl; R^3 = i-Pr

Figure 3.1.1: Synthesis of 3-(Nitrooxyalkyl or 4-chlorobutyl) 5-Alkyl 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**6-17**)

3.1.1.1 4-Bromo-1-butanol (1c)

4-Bromo-1-butanol (**1c**) was prepared by modification of previously reported procedures [Wälchli & Eugster, 1978; Vedejs *et al.*, 1979]. Concentrated sulfuric acid (1 mL), and then HBr (16 mL of 48% w/v, 0.14 mol of HBr), were added dropwise to THF (10 g, 0.14 mol) at 0°C with stirring, and the reaction mixture was refluxed for 90 minutes. After cooling to 0°C, the reaction mixture was neutralized with $NaHCO_3$, and water (50 mL) was added. The alcohol product was extracted with diethyl ether (3 × 60 mL), washed with brine, and the organic fraction was dried ($MgSO_4$). Removal of the

solvent *in vacuo* afforded **1c** (6.3 g, 30%, oil) that was used for the synthesis of 4-bromobutyl acetoacetate (**2c**).

3.1.1.2 General Method for the Synthesis of Haloalkyl Acetoacetates (2a-e)

Bromoalkyl acetoacetates (**2a-e**) were prepared by modification of previously reported procedures [Clemens & Hyatt, 1985; Iqbal & Knaus, 1996]. A solution of freshly distilled 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (7.1 g, 50 mmol) and the haloalkyl alcohol (50 mmol: **1a**, 6.3 g; **1b**, 7.0 g; **1c**, 7.7 g; **1d**, 10.9 g; **1e**, 5.5 g) in xylene (6 mL) was refluxed for 60 minutes. Removal of the solvent *in vacuo* gave a residue that was purified by vacuum distillation to afford the respective oily bromoalkyl acetoacetate (**2a**, 129°C / 1.6 mm Hg, 3.5 g, 34%; **2b**, 95°C / 1.6 mm Hg, 3.1 g, 27%; **2c**, 152°C / 1.6 mm Hg, 3.8 g, 32%; **2d**, 170°C / 1.6 mm Hg, 13.4 g, 89%; **2e**, 102°C / 1.6 mm Hg, 1.3 g, 13%). Products **2a-d** were used for the preparation of nitrooxyalkyl acetoacetates (**3a-d**), and **2e** was subsequently used for the synthesis of 3-isopropyl 5-(4-chlorobutyl) 1,4-dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**17**).

3.1.1.3 General Method for the Synthesis of Nitrooxyalkyl Acetoacetates (3a-d)

A solution of the haloalkyl acetoacetate (10 mmol: **2a**, 2.1 g; **2b**, 2.2 g; **2c**, 2.4 g; **2d**, 3.0 g) containing $AgNO_3$ (**2a-c**, 2.0 g, 12 mmol; **2d**, 4.1 g, 24 mmol) in acetonitrile (20 mL) was stirred in the dark at 22°C for 5 days. Removal of the solvent *in vacuo* gave a residue that was filtered *in vacuo* through a 5 cm layer of silica gel using dichloromethane as the transfer solvent. Removal of the solvent *in vacuo* gave a residue that was purified by silica gel column chromatography using either dichloromethane or dichloromethane-hexane or ethyl acetate-hexane as eluent. The respective oily nitrooxyalkyl acetoacetates (**3a**, 1.9 g, 72%; **3b**, 1.2 g, 60%; **3c**, 1.4 g, 66%; **3d**, 0.6 g, 24%) were used directly for synthesis of the 3-nitrooxyalkyl 5-alkyl 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**6-16**).

3.1.1.4 General Method for the Synthesis of 3-Nitrooxyalkyl 5-Alkyl 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (6-16), and 3-Isopropyl 5-(4-Chlorobutyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (17).

A solution of the nitrooxyalkyl acetoacetate (2 mmol: **3a**, 382 mg; **3b**, 410 mg; **3c**, 438 mg; **3d**, 532 mg), or **2e** (812 mg, 2 mmol), alkyl 3-aminocrotonate (2 mmol: **5a**, 258 mg; **5b**, 286 mg; **5c** or **5d**, 314 mg), and a pyridinecarboxaldehyde (**4a**, **4b** or **4c**, 214 mg, 2 mmol) in isopropanol (10 mL) was stirred at 22°C for 1 to 3 days. Removal of the solvent *in vacuo* gave a residue that was partially purified PTLC using either dichloromethane or dichloromethane-hexane or ethyl acetate-hexane, as eluant. The residue obtained was further purified by preparative thin layer chromatography using either dichloromethane or dichloromethane-hexane or ethyl acetate-hexane or THF-hexane, as development solvent. The TLC band containing the product was extracted with methanol, and the solvent was removed *in vacuo*. The residue obtained was dissolved in dichloromethane, filtered through a cotton plug, the solvent was removed *in vacuo*, and the solid product was recrystallized at least twice from a suitable two-solvent mixture (dichloromethane or ethyl acetate, and either diethyl ether or hexane). The percent yield, melting point, spectral and analytical data for products **6-17** are listed below.

3.1.1.4.1 3-(2-Nitrooxyethyl) 5-Ethyl 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (6)

Yield: 32% (solid); mp 134°C; IR ($CHCl_3$): 3342 (NH), 1697 (C=O), 1636, 1280 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.21 (t, J = 7.0 Hz, 3H, CH_2CH_3), 2.22 and 2.23 (two s, 3H each, C-2 and C-6 *Me's*), 4.06 (q, J = 7.0 Hz, 2H, CH_2CH_3), 4.22-4.38 (m, 2H, CH_2ONO_2), 4.54-4.62 (m, 2H, $CO_2CH_2CH_2ONO_2$), 5.17 (s, 1H, H-4), 7.17 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.6 Hz, 1H, pyridinyl H-5), 7.40 (d, $J_{3,4}$ = 7.9 Hz, 1H, pyridinyl H-3), 7.61 (dd, $J_{3,4}$ = 7.9, $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.48 (d, $J_{5,6}$ = 4.6 Hz, 1H, pyridinyl H-6), 9.44 (br s, 1H, NH). *Anal.* Calcd. for $C_{18}H_{21}N_3O_7 \bullet 1/6H_2O$: C 54.82, H 5.45, N 10.65. Found: C 55.06, H 5.14, N 10.25.

3.1.1.4.2 3-Isopropyl 5-(2-Nitrooxyethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (7)

Yield: 8% (solid); mp 160°C; IR ($CHCl_3$): 3344 (NH), 1691 (C=O), 1642, 1280 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.11 and 1.23 (two d, $J_{CH,Me}$ = 6.1 Hz, 3H each, $CHMe_2$), 2.25 (s, 6H, C-2 and C-6 *Me's*), 4.22-4.38 (m, 2H, CH_2ONO_2), 4.52-4.68 (m, 2H, $CO_2CH_2CH_2ONO_2$), 4.94 (septet, $J_{CH,Me}$ = 6.1 Hz, 1H, $CHMe_2$), 5.15 (s, 1H, H-4), 7.15 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl-5), 7.38 (d, $J_{3,4}$ = 7.9 Hz, 1H, pyridinyl H-3), 7.58 (dd, $J_{3,4}$ = 7.9, $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.48 (d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-6), 9.10 (br s, 1H, NH). *Anal.* Calcd. for $C_{19}H_{23}N_3O_7 \bullet 2/5H_2O$: C 55.31, H 5.81, N 10.18. Found: C 55.69, H 5.62, N 9.23.

3.1.1.4.3 3-Isopropyl 5-(2-Nitrooxyethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (8)

Yield: 17% (solid); mp 135°C; IR ($CHCl_3$): 3344 (NH), 1695 (C=O), 1641, 1281 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.11 and 1.25 (two d, $J_{CH,Me}$ = 6.1 Hz, 3H each, $CHMe_2$), 2.33 (s, 6H, C-2 and C-6 *Me's*), 4.28-4.38 (m, 2H, CH_2ONO_2), 4.56-4.68 (m, 2H, $CO_2CH_2CH_2ONO_2$), 4.93 (s, 1H, H-4), 4.99 (septet, $J_{CH,Me}$ = 6.1 Hz, 1H, $CHMe_2$), 6.52 (sharp s, 1H, NH), 7.17 (dd, $J_{4,5}$ = 7.9, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-5), 7.61 (d, $J_{4,5}$ = 7.9 Hz, 1H, pyridinyl H-4), 8.37 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6), 8.52 (s, 1H, pyridinyl H-2). *Anal.* Calcd. for $C_{19}H_{23}N_3O_7 \bullet 3/8H_2O$: C 55.37, H 5.81, N 10.20. Found: C 54.97, H 5.30, N 9.90.

3.1.1.4.4 3-Isopropyl 5-(2-Nitrooxyethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (9)

Yield: 41% (solid); mp 173°C; IR ($CHCl_3$): 3359 (NH), 1695 (C=O), 1641, 1281 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.12 and 1.25 (two d, $J_{CH,Me}$ = 6.4 Hz, 3H each, $CHMe_2$), 2.35 (s, 6H, C-2 and C-6 *Me's*), 4.28-4.40 (m, 2H, CH_2ONO_2), 4.53-4.70 (m, 2H, $CO_2CH_2CH_2ONO_2$), 4.90-5.14 (m, 2H, $CHMe_2$, H-4), 6.52 (sharp s, 1H, NH), 7.22 (d, $J_{2,3}$ = $J_{5,6}$ = 4.6 Hz, 2H, pyridinyl H-3 and H-5), 8.44 (d, $J_{2,3}$ = $J_{5,6}$ = 4.6 Hz, 2H, pyridinyl H-2 and H-6). *Anal.* Calcd. for $C_{19}H_{23}N_3O_7 \bullet 3/8H_2O$: C 55.7[illegible].77, N 10.26. Found: C 55.99, H 5.45, N 9.86.

3.1.1.4.5 3-(2-Nitrooxyethyl) 5-Isobutyl 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (10)

Yield: 33% (solid); mp 126°C; IR ($CHCl_3$): 3344 (NH), 1695 (C=O), 1642, 1280 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.86 and 0.89 (two d, $J_{CH,Me}$ = 6.7 Hz, 3H each, $CHMe_2$), 1.80-2.00 (m, 1H, CH_2CHMe_2), 2.22 and 2.23 (two s, 3H each, C-2 and C-6 *Me's*), 3.81 (d, $J_{CH,CH2}$ = 6.7 Hz, 2H, CO_2CH_2CH), 4.12-4.40 (m, 2H, $CO_2CH_2CH_2ONO_2$), 4.55-4.67 (m, 2H, $CO_2CH_2CH_2ONO_2$), 5.20 (s, 1H, H-4), 7.17 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-5), 7.42 (d, $J_{3,4}$ = 7.6 Hz, 1H, pyridinyl H-3), 7.62 (dd, $J_{3,4}$ = $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.48 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6), 9.55 (br s, 1H, NH). *Anal.* Calcd. for $C_{20}H_{25}N_3O_7$: C 57.27, H 6.01, N 10.02. Found: C 57.80, H 5.96, N 9.93.

3.1.1.4.6 3-tert-Butyl 5-(2-Nitrooxyethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (11)

Yield: 14% (solid); mp 132°C; IR ($CHCl_3$): 3343 (NH), 1695 (C=O), 1637, 1279 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.37 (s, 9H, CMe_3), 2.19 and 2.23 (two s, 3H each, C-2 and C-6 *Me's*), 4.20-4.40 (m, 2H, CH_2ONO_2), 4.52-4.68 (m, 2H, $CO_2CH_2CH_2ONO_2$), 5.11 (s, 1H, H-4), 7.15 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-5), 7.39 (d, $J_{3,4}$ = 7.9 Hz, 1H, pyridinyl H-3), 7.61 (dd, $J_{3,4}$ = 7.9, $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.48 (d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-6), 9.05 (br s, 1H, NH). *Anal.* Calcd. for $C_{20}H_{25}N_3O_7$: C 57.27, H 6.01, N 10.02. Found: C 57.21, H 5.93, N 9.88.

3.1.1.4.7 3-Isopropyl 5-(3-Nitrooxypropyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (12)

Yield: 43% (solid); mp 139°C; IR ($CHCl_3$): 3344 (NH), 1692 (C=O), 1639, 1281 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.11 and 1.22 (two d, $J_{CH,Me}$ = 6.4 Hz, 3H each, $CHMe_2$), 1.96-2.10 (m, 2H, $CH_2CH_2CH_2$), 2.22 and 2.24 (two s, 3H each, C-2 and C-6 *Me's*), 4.02-4.22 (m, 2H, CH_2ONO_2), 4.30-4.44 (m, 2H, $CO_2CH_2CH_2ONO_2$), 4.95 (septet, $J_{CH,Me}$ = 6.4 Hz, 1H, $CHMe_2$), 5.16 (s, 1H, H-4), 7.15 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-5), 7.38 (d, $J_{3,4}$ = 7.9 Hz, 1H, pyridinyl H-3), 7.60 (dd, $J_{3,4}$ = 7.9, $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.48 (d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-6), 8.93 (br s, 1H, NH). *Anal.* Calcd. for $C_{20}H_{25}N_3O_7$: C 57.27, H 6.01, N 10.02. Found: C 57.44, H 5.98, N 9.70 (Figure 3.1.1.4.7).

3.1.1.4.8 3-Isopropyl 5-(4-Nitrooxybutyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (13)

Yield: 26% (solid); mp 135°C; IR ($CHCl_3$): 3343 (NH), 1684 (C=O), 1653, 1278 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.11 and 1.21 (two d, $J_{CH,Me}$ = 6.1 Hz, 3H each, $CHMe_2$), 1.62-1.76 (m, 4H, $CH_2CH_2CH_2CH_2$), 2.23 and 2.24 (two s, 3H each, C-2 and C-6 *Me's*), 3.94-4.14 (m, 2H, CH_2ONO_2), 4.33-4.44 (m, 2H, $CO_2CH_2CH_2ONO_2$), 4.95 (septet, $J_{CH,Me}$ = 6.1 Hz, 1H, $CHMe_2$), 5.16 (s, 1H, *H-4*), 7.14 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.2 Hz, 1H, pyridinyl H-5), 7.38 (d, $J_{3,4}$ = 7.6 Hz, 1H, pyridinyl H-3), 7.59 (dd, $J_{3,4}$ = $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.48 (d, $J_{5,6}$ = 4.2 Hz, 1H, pyridinyl H-6), 8.77 (sharp s, 1H, N*H*). *Anal.* Calcd. for $C_{21}H_{27}N_3O_7 \bullet 1/5H_2O$: C 57.71, H 6.32, N 9.61. Found: C 58.05, H 6.19, N 9.21.

3.1.1.4.9 3-(1,3-Dinitrooxy-2-propyl) 5-Isopropyl 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (14)

Yield: 31% (solid); mp 101°C; IR ($CHCl_3$): 3328 (NH), 1694 (C=O), 1647, 1275 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.13 and 1.23 (two d, $J_{CH,Me}$ = 6.4 Hz, 3H each, $CHMe_2$), 2.24 and 2.26 (two s, 3H each, C-2 and C-6 *Me's*), 4.38 (dd, J_{gem} = 12.0, J_{vic} = 5.4 Hz, 1H, $CH_aH_bONO_2$), 4.50 (dd, J_{gem} = 12.0, J_{vic} = 4.3 Hz, 1H, $CH_aH_bONO_2$), 4.60 (dd, J_{gem} = 12.0, J_{vic} = 5.4 Hz, 1H, $CH_aH_bONO_2$), 4.73 (dd, J_{gem} = 12.0, J_{vic} = 4.3 Hz, 1H, $CH_aH_bONO_2$), 4.95 (septet, $J_{CH,Me}$ = 6.4 Hz, 1H, $CHMe_2$), 5.13 (s, 1H, H-4), 5.32-5.40 [m, 1H, $CH(CH_2ONO_2)_2$], 7.19 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-5), 7.39 (d, $J_{3,4}$ = 7.6 Hz, 1H, pyridinyl H-3), 7.63 (dd, $J_{3,4}$ = $J_{5,6}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.50 (d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-6), 9.25 (sharp s, 1H, N*H*). *Anal.* Calcd. for $C_{20}H_{24}N_4O_{10} \bullet H_2O$: C 48.19, H 5.26, N 11.24. Found: C 48.42, H 5.14, N 9.05.

3.1.1.4.10 3-(1,3-Dinitrooxy-2-propyl) 5-Isopropyl 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (15)

Yield: 16% (solid); mp 117°C; IR (acetone): 3338 (NH), 1694 (C=O), 1643, 1274 (ONO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.09 and 1.23 (two d, $J_{CH,Me}$ = 6.4 Hz, 3H each, $CHMe_2$), 2.34 and 2.36 (two s, 3H each, C-2 and C-6 *Me's*), 4.40 (dd, J_{gem} = 12.0, J_{vic} =

5.4 Hz, 1H, C*H*$_a$H$_b$ONO$_2$), 4.50 (dd, J_{gem} = 12.0, J_{vic} = 4.3 Hz, 1H, CH$_a$*H*$_b$ONO$_2$), 4.62 (dd, J_{gem} = 12.0, J_{vic} = 5.4 Hz, 1H, C*H*$_a$H$_b$ONO$_2$), 4.72 (dd, J_{gem} = 12.0, J_{vic} = 4.3 Hz, 1H, CH$_a$*H*$_b$ONO$_2$), 4.93 (s, 1H, H-4), 4.94 (septet, $J_{CH,Me}$ = 6.4 Hz, 1H, C*H*Me$_2$), 5.30-5.40 [m, 1H, C*H*(CH$_2$ONO$_2$)$_2$], 6.20 (sharp s, 1H, N*H*), 7.18 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-5), 7.64 (d, $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.30 (d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-6), 8.52 (s, 1H, pyridinyl H-2). *Anal.* Calcd. for C$_{20}$H$_{24}$N$_4$O$_{10}$•4/9H$_2$O: C 49.18, H 5.14, N 11.47. Found: C 49.59, H 4.81, N 10.15.

3.1.1.4.11 3-(1,3-Dinitrooxy-2-propyl) 5-Isopropyl 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (16)

Yield: 7% (solid); mp 125°C; IR (acetone): 3337 (NH), 1692 (C=O), 1642, 1274 (ONO_2) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.12 and 1.23 (two d, $J_{CH,Me}$ = 6.1 Hz, 3H each, CH*Me*$_2$), 2.34 and 2.36 (two s, 3H each, C-2 and C-6 *Me's*), 4.40 (dd, J_{gem} = 12.0, J_{vic} = 5.4 Hz, 1H, C*H*$_a$H$_b$ONO$_2$), 4.52 (dd, J_{gem} = 12.0, J_{vic} = 4.3 Hz, 1H, CH$_a$*H*$_b$ONO$_2$), 4.62 (dd, J_{gem} = 12.0, J_{vic} = 5.4 Hz, 1H, C*H*$_a$H$_b$ONO$_2$), 4.73 (dd, J_{gem} = 12.0, J_{vic} = 4.3 Hz, 1H, CH$_a$*H*$_b$ONO$_2$), 4.90 (s, 1H, H-4), 4.98 (septet, $J_{CH,Me}$ = 6.1 Hz, 1H, C*H*Me$_2$), 5.50-5.60 [m, 1H, C*H*(CH$_2$ONO$_2$)$_2$], 5.85 (sharp s, 1H, N*H*), 7.20 (d, $J_{2,3}$ = $J_{5,6}$ = 4.6 Hz, 2H, pyridinyl H-3 and H-5), 8.41 (d, $J_{2,3}$ = $J_{5,6}$ = 4.6 Hz, 2H, pyridinyl H-2 and H-6). *Anal.* Calcd. for C$_{20}$H$_{24}$N$_4$O$_{10}$•1/5H$_2$O: C 49.63, H 5.08, N 11.57. Found: C 50.01, H 5.04, N 10.71.

3.1.1.4.12 3-Isopropyl 5-(4-Chlorobutyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (17)

Yield: 39% (solid); mp 136°C; IR ($CHCl_3$): 3361 (NH), 1685 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.10 and 1.22 (two d, $J_{CH,Me}$ = 6.1 Hz, 3H each, CH*Me*$_2$), 1.66-1.85 (m, 4H, CH$_2$C*H*$_2$C*H*$_2$CH$_2$Cl), 2.23 and 2.24 (two s, 3H each, C-2 and C-6 *Me's*), 3.51 (t, J = 6.1 Hz, 2H, CH$_2$C*H*$_2$Cl), 3.98-4.16 (m, 2H, CO$_2$C*H*$_2$CH$_2$CH$_2$Cl), 4.94 (sept, $J_{CH,Me}$ = 6.1 Hz, 1H, C*H*Me$_2$), 5.17 (s, 1H, H-4), 7.15 (dd, $J_{4,5}$ = 7.6, $J_{5,6}$ = 4.3 Hz, 1H, pyridinyl H-5), 7.41 (d, $J_{3,4}$ = 7.9 Hz, 1H, pyridinyl H-3), 7.60 (dd, $J_{3,4}$ = 7.9, $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.48 (d, $J_{5,6}$ = 4.3 Hz, 1H, pyridinyl H-6), 9.03 (br s, 1H, N*H*). *Anal.* Calcd. for C$_{21}$H$_{27}$N$_2$O$_4$Cl: C 61.99, H 6.69, N 6.88. Found: C 62.17, H 6.86, N 6.85.

3.1.2 1,4-Dihydropyridine-Furoxan Hybrids

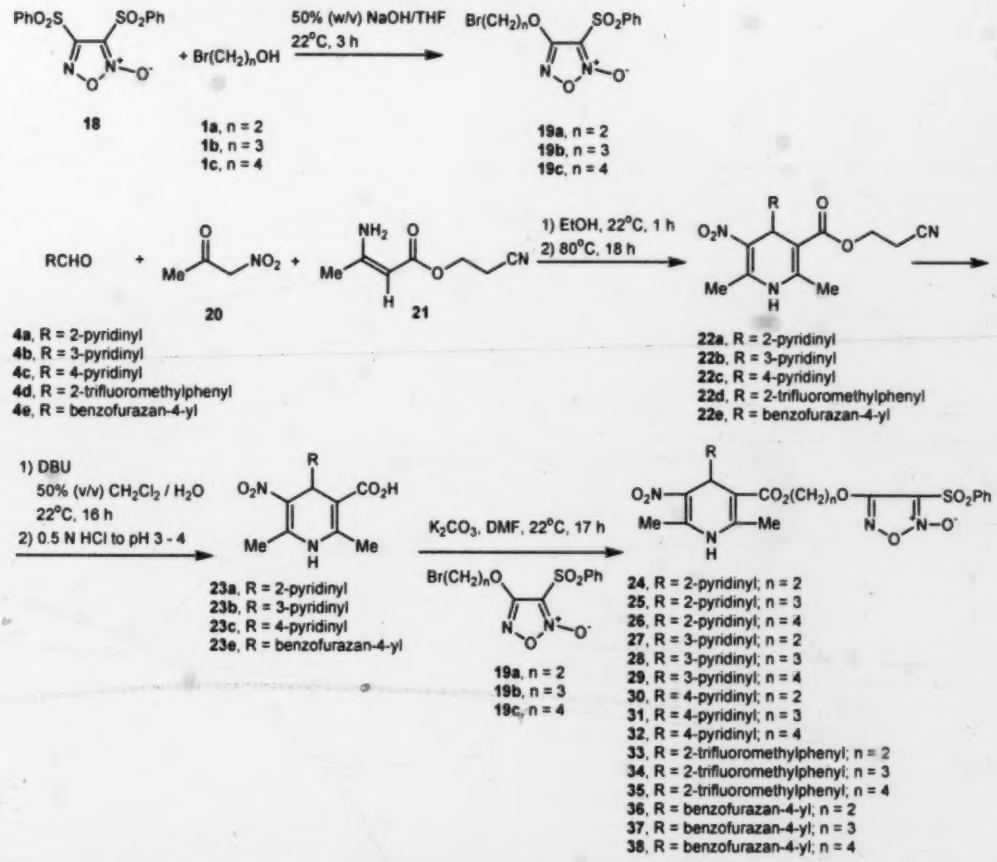

50% (w/v) NaOH/THF
22°C, 3 h
+ Br(CH2)nOH
18
1a, n = 2
1b, n = 3
1c, n = 4
19a, n = 2
19b, n = 3
19c, n = 4
RCHO
20
21
1) EtOH, 22°C, 1 h
2) 80°C, 18 h
4a, R = 2-pyridinyl
4b, R = 3-pyridinyl
4c, R = 4-pyridinyl
4d, R = 2-trifluoromethylphenyl
4e, R = benzofurazan-4-yl
22a, R = 2-pyridinyl
22b, R = 3-pyridinyl
22c, R = 4-pyridinyl
22d, R = 2-trifluoromethylphenyl
22e, R = benzofurazan-4-yl
1) DBU
50% (v/v) CH2Cl2 / H2O
22°C, 16 h
2) 0.5 N HCl to pH 3 - 4
23a, R = 2-pyridinyl
23b, R = 3-pyridinyl
23c, R = 4-pyridinyl
23e, R = benzofurazan-4-yl
K2CO3, DMF, 22°C, 17 h
19a, n = 2
19b, n = 3
19c, n = 4
24, R = 2-pyridinyl; n = 2
25, R = 2-pyridinyl; n = 3
26, R = 2-pyridinyl; n = 4
27, R = 3-pyridinyl; n = 2
28, R = 3-pyridinyl; n = 3
29, R = 3-pyridinyl; n = 4
30, R = 4-pyridinyl; n = 2
31, R = 4-pyridinyl; n = 3
32, R = 4-pyridinyl; n = 4
33, R = 2-trifluoromethylphenyl; n = 2
34, R = 2-trifluoromethylphenyl; n = 3
35, R = 2-trifluoromethylphenyl; n = 4
36, R = benzofurazan-4-yl; n = 2
37, R = benzofurazan-4-yl; n = 3
38, R = benzofurazan-4-yl; n = 4

Figure 3.1.2: Synthesis of 5-[(3-Benzenesulfonyl)furoxan-4-yloxy]alkyl 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl, benzofurazan-4-yl, 2-, 3- or 4-pyridinyl)-5-pyridinecarboxylates (**24-38**)

3.1.2.1 General Method for the Synthesis of 3-Benzenesulfonyl-4-(bromoalkoxy)furoxans (19a-c)

A solution of 50% (w/v) sodium hydroxide (2.85 g, 71.3 mmol) was added in aliquots to a mixture of bis(benzenesulfonyl)furoxan (**18**) (4.0 g, 10.9 mmol) and bromoalkyl alcohol (23.8 mmol: **1a**, 2.97 g; **1b**, 3.31 g; **1c**, 3.64 g) in THF (40 mL) at 22°C with stirring, and the reaction was allowed to proceed for 3 hours prior to pouring onto ice-water (500 mL). The precipitate was filtered, dried, and recrystallized from

ethanol. The percent yield, melting point, spectral and analytical data for products **19a-c** are listed below.

3.1.2.1.1 3-Benzenesulfonyl-4-(2-bromoethoxy)furoxan (19a)

Yield: 99% (solid); mp 118-119°C; H^1 NMR ($CDCl_3$): δ 3.73 (t, *J* = 5.8 Hz, 2H, $BrCH_2$), 4.74 (t, *J* = 5.8 Hz, 2H, CH_2O), 7.63 (d, *J* = 7.9 Hz of d, *J* = 7.9 Hz, 2H, phenyl H-3, H-5), 7.76 (d, *J* = 7.9 Hz of d, *J* = 7.9 Hz, 1H, phenyl H-4), 8.09 (d, *J* = 7.9 Hz, 2H, phenyl H-2, H-6). *Anal.* Calcd. for $C_{10}H_9BrN_2O_5S$: C 34.40, H 2.60, N 8.02. Found: C 34.72, H 2.86, N 7.95.

3.1.2.1.2 3-Benzenesulfonyl-4-(3-bromopropoxy)furoxan (19b)

Yield: 89% (solid); mp 105-106°C (EtOH); ^1H NMR ($CDCl_3$): δ 2.41 (quintet, *J* = 6.4 Hz, 2H, $CH_2CH_2CH_2$), 3.60 (t, *J* = 6.4 Hz, 2H, $BrCH_2$), 4.59 (t, *J* = 6.4 Hz, 2H, CH_2O), 7.41 (d, *J* = 7.9 Hz of d, *J* = 7.9 Hz, 2H, phenyl H-3, H-5), 7.78 (d, *J* = 7.9 Hz of d, *J* = 7.9 Hz, 1H, phenyl H-4), 8.06 (d, *J* = 7.9 Hz, 2H, phenyl H-2, H-6). *Anal.* Calcd. for $C_{11}H_{11}BrN_2O_5S$: C 36.38, H 3.05, N 7.71. Found: C 36.19, H 2.98, N 7.67.

3.1.2.1.3 3-Benzenesulfonyl-4-(4-bromobutoxy)furoxan (19c)

Yield: 78% (solid); mp 64-65°C (EtOH); ^1H NMR ($CDCl_3$): δ 2.01-2.10 (m, 4H, $CH_2CH_2CH_2CH_2$), 3.46-3.53 (m, 2H, CH_2Br), 4.44-4.49 (m, 2H, CH_2O), 7.62 (d, *J* = 7.9 Hz of d, *J* = 7.9 Hz, 2H, phenyl H-3, H-5), 7.70 (d, *J* = 7.9 Hz of d, *J* = 7.9 Hz, 1H, phenyl H-4), 8.96 (d, *J* = 7.9 Hz, 2H, phenyl H-2, H-6). *Anal.* Calcd. for $C_{12}H_{13}BrN_2O_5S$: C 38.21, H 3.47, N 7.43. Found: C 38.49, H 3.46, N 7.43.

3.1.2.2 General Method for the Synthesis of 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl, benzofurazan-4-yl, 2-, 3- or 4-pyridinyl)-5-pyridinecarboxylates (22a-e)

A solution of nitroacetone (**20**) (4.78 g, 46.4 mmol) in ethanol (50 mL) was added drop-wise to a solution of aryl or heteroaryl carboxaldehyde (**4a-e**) (35.6 mmol: **4a-c**, 3.82 g; **4d**, 6.20 g; **4e**, 5.27 g) in ethanol (30 mL) at 0°C with stirring. This mixture was stirred at 22°C for 20 minutes and then 2-cyanoethyl 3-aminocrotonate (5.0 g, 32.4

mmol) was added. The reaction was allowed to proceed at 22°C for 1 hour and then at 80°C for 18 hours with stirring. The solvent was partially removed *in vacuo* and the solution was poured onto ice-water (50 mL). The solid was filtered, dried, and separated by silica gel column chromatography using ethyl acetate-hexane at a suitable mixture ratio as eluant. The solid was recrystallized from ethyl acetate-diethyl ether. The percent yield, melting point, spectral and analytical data for products **22a-e** are listed below.

3.1.2.2.1 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-pyridinyl)-5-pyridinecarboxylate (22a)

Yield: 7% (solid); mp 194-195°C; ^{1}H NMR ($CDCl_3$): δ 2.28 (s, 3H, C-2 *Me*), 2.45 (s, 3H, C-6 *Me*), 2.65 (t, J = 6.1 Hz, 2H, CH_2CH_2CN), 4.23-4.31 (m, 2H, CH_2CH_2CN), 5.61 (s, 1H, H-4), 7.30 (d, $J_{4,5}$= 7.3 Hz of d, $J_{5,6}$= 4.3 Hz, 1H, pyridinyl H-5), 7.69 (d, $J_{3,4}$= 7.9 Hz, 1H, pyridinyl H-3), 7.77 (d, $J_{3,4}$= 7.9 Hz of d, $J_{4,5}$= 7.3 Hz, 1H, pyridinyl H-4), 8.49 (d, $J_{5,6}$ = 4.3 Hz, 1 H, pyridinyl H-6), 9.95 (s, 1H, N*H*). *Anal.* Calcd. for $C_{16}H_{16}N_4O_4$: C 58.53, H 4.91, N 17.06. Found: C 58.56, H 4.71, N 16.82.

3.1.2.2.2 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(3-pyridinyl)-5-pyridinecarboxylate (22b)

Yield: 36% (solid); mp 220°C; ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, C-2 *Me*), 2.50 (s, 3H, C-6 *Me*), 2.83-2.88 (m, 2H, CH_2CH_2CN), 4.13-4.18 (m, 2H, OCH_2CH_2), 5.21 (s, 1H, H-4), 7.29 (d, $J_{4,5}$= 7.9 Hz of d, $J_{5,6}$= 4.6 Hz, 1H, pyridinyl H-5), 7.61 (d, $J_{4,5}$ = 7.9 Hz of d, $J_{4,6}$ = 1.8 Hz, 1H, pyridinyl H-4), 8.35 (d, $J_{5,6}$ = 4.58 Hz of d, $J_{4,6}$ = 1.8 Hz, 1H, pyridinyl H-6), 8.45 (s, 1H, pyridinyl H-2), 9.76 (s, 1H, N*H*). *Anal.* Calcd. for $C_{16}H_{16}N_4O_4 \cdot 1/3H_2O$: C 57.48, H 5.02, N 16.76. Found: C 57.80, H 5.13, N 16.38.

3.1.2.2.3 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(4-pyridinyl)-5-pyridinecarboxylate (22c)

Yield: 22% (solid); mp 186-187°C (ethyl acetate-hexane); H^1 NMR (DMSO-d_6): δ 2.34 (s, 3H, C-2 *Me*), 2.50 (s, 3H, C-6 *Me*), 2.76-2.81 (m, 2H, CH_2CH_2CN), 4.18 (t, J = 6.0 Hz, 2H, OCH_2CH_2), 5.24 (s, 1H, H-4), 7.20 (d, $J_{2,3}$ = $J_{5,6}$ = 6.1 Hz, 2H, pyridinyl H-3,

H-5), 8.40 (d, $J_{2,3} = J_{5,6}$ = 6.1 Hz, 2H, pyridinyl H-2, H-6), 9.61 (s, 1H, N*H*). *Anal.* Calcd. for $C_{16}H_{16}N_4O_4$: C 58.53, H 4.91, N 17.06. Found: C 58.34, H 4.79, N 16.74.

3.1.2.2.3 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyphenyl)-5-pyridinecarboxylate (22d)

Yield: 67% (solid); mp 168-169°C; ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, C-2 *Me*), 2.47 (s, 3H, C-6 *Me*), 2.79 (t, *J* = 6.1 Hz, 2H, $CH_2C\underline{H_2}CN$), 4.01-4.09 (m, 1H, C*H*H'CH_2CN), 4.16-4.24 (m, 1H, CH*H*'CH_2CN),), 5.78 (s, 1H, H-4), 7.37 (d, $J_{3,4}$ = 7.3 Hz of d, $J_{4,5}$ = 7.3 Hz, 1H, phenyl H-4), 7.45 (d, $J_{5,6}$ = 7.3 Hz, 1H, phenyl H-6), 7.50-7.62 (m, 2H, phenyl H-3, H-5), 9.66 (s, 1H, NH). *Anal.* Calcd. for $C_{18}H_{16}F_3N_3O_4$: C 54.69, H 4.08, N 10.63. Found: C 54.57, H 3.99, N 10.54.

3.1.2.2.4 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)-5-pyridinecarboxylate (22e)

Yield: 26% (solid); mp 171-172°C; ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H, C-2 *Me*), 2.56 (s, 3H, C-6 *Me*), 2.67 (t, *J* = 6.2 Hz, 2H, CH_2CN), 4.29 (m, 2H, CO_2CH_2), 5.82 (s, 1H, H-4), 6.82 (s, 1H, N*H*), 7.38 (dd, *J* = 8.8, 6.4 Hz, 1H, benzofurazanyl H-6), 7.52 (d, *J* = 6.4 Hz, 1H, benzofurazanyl H-5), 7.70 (d, *J* = 8.8 Hz, 1H, benzofurazanyl H-7). Due to its instability, **22e** was subsequently used for the synthesis of **23e**.

3.1.2.3 General Method for the Synthesis of 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl, benzofurazan-4-yl, 2-, 3- or 4-pyridinyl)-5-pyridinecarboxylic Acids (23a-e)

A mixture of the 2-cyanoethyl ester selected (**22a-e**) (3.7 mmol: **22a-c**, 1.21 g; **22d**, 1.46 g; **22e**, 1.37 g) and DBU (1.67 g, 11.0 mmol) in dichloromethane (25 mL) and water (25 mL) was stirred at 22°C for 16 hours, and the reaction mixture was poured onto ice-water (50 mL). The pH of the water phase was adjusted to 3 – 4 using HCl (0.5 N) until a solid precipitated out of solution. The solid was filtered, washed with diethyl ether (3 ×10 mL) and then recrystallized from methanol-diethyl ether. The percent yield, melting point, spectral and analytical data for products **23a-e** are listed below.

3.1.2.3.1 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-pyridinyl)-5-pyridinecarboxylic Acid (23a)

Yield: 50% (solid); mp 212-213°C; ^{1}H NMR (DMSO-d_6): δ 2.24 (s, 3H, C-2 *Me*), 2.49 (s, 3H, C-6 *Me*), 5.35 (s, 1H, H-4), 7.17 (d, $J_{4,5}$ = 7.6 Hz of d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-5), 7.27 (d, $J_{3,4}$ = 7.6 Hz, 1H, pyridinyl H-3), 7.66 (d, $J_{3,4}$ = 7.6 Hz of d, $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.41 (d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-6), 9.49 (s, 1H, N*H*), 12.20 (br s, 1H, COO*H*). *Anal.* Calcd. for $C_{13}H_{13}N_3O_4$: C 56.72, H 4.76, N 15.27. Found: C 56.66, H 4.70, N 14.97.

3.1.2.3.2 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(3-pyridinyl)-5-pyridinecarboxylic Acid (23b)

Yield: 40% (solid); mp 212-213°C; ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, C-2 *Me*), 2.50 (s, 3H, C-6 *Me*), 5.20 (s, 1H, H-4), 7.28 (d, $J_{4,5}$ = 7.9 Hz of d, $J_{5,6}$ = 4.0 Hz, 1H, pyridinyl H-5), 7.55 (d, $J_{4,5}$ = 7.9 Hz, 1H, pyridinyl H-4), 8.35 (d, $J_{5,6}$ = 4.0 Hz of d, $J_{2,4}$ = 1.8 Hz, 1H, pyridinyl H-6), 8.41 (d, $J_{2,4}$ = 1.8 Hz, 1H, pyridinyl H-2), 9.76 (s, 1H, N*H*), 12.35 (br s, 1H, COO*H*). *Anal.* Calcd. for $C_{13}H_{13}N_3O_4$: C 56.72, H 4.76, N 15.27. Found: C 56.50, H 4.78, N 15.50.

3.1.2.3.3 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(4-pyridinyl)-5-pyridinecarboxylic Acid (23c)

Yield: 83% (solid); mp: 168-169°C; ^{1}H NMR (DMSO-d_6): δ 2.27 (s, 3H, C-2 *Me*), 2.50 (s, 3H, C-6 *Me*), 5.23 (s, 1H, H-4), 7.17 (d, $J_{2,3}$ = $J_{5,6}$ = 5.8 Hz, 2H, pyridinyl H-3, H-5), 8.43 (d, $J_{2,3}$ = $J_{5,6}$ = 5.8 Hz, 2H, pyridinyl H-2, H-6), 9.61 (s, 1H, N*H*), 12.35 (br s, 1H, COO*H*). *Anal.* Calcd. for $C_{13}H_{13}N_3O_4$: C 56.72, H 4.76, N 15.27. Found: C 56.66, H 4.66, N 14.98.

3.1.2.3.4 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-5-pyridinecarboxylic Acid (23d)

Yield: 97% (solid); mp 194-195°C [lit. mp 204-205°C, Shan *et al.*, 2002]; ^{1}H NMR and *Anal.* Calcd. data are similar to those reported by Shan *et al.* (2002).

3.1.2.3.5 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)-5-pyridinecarboxylic Acid (23e)

Yield: 53% (solid); mp 210 °C; ^{1}H NMR (DMSO-d_6): δ 2.27 (s, 3H, C-2 *Me*), 2.50 (s, 3H, C-6 *Me*), 5.70 (s, 1H, H-4), 7.40 (d, *J* = 6.7 Hz, 1H, benzofurazanyl H-5), 7.55 (dd, *J* = 9.2, 6.7 Hz, 1H, benzofurazanyl H-6), 7.88 (d, *J* = 9.2 Hz, 1H, benzofurazanyl H-7), 9.82 (s, 1H, N*H*), 12.29 (br s, 1H, COO*H*). *Anal.* Calcd. for $C_{14}H_{12}N_4O_5$: C 53.17, H 3.82, N 17.71. Found: C 53.19, H 3.76, N 17.40.

3.1.2.4 General Method for the Synthesis of 5-[(3-Benzenesulfonyl)furoxan-4-yloxy]alkyl 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl, 4-benzofurazanyl, 2-, 3- or 4-pyridinyl)-3-pyridinecarboxylates (24-38)

A solution of the carboxylic acid selected (**23a-e**) (1.65 mmol: **23a-c**, 454 mg; **23d**, 564 mg; **23e**, 522 mg), the 3-benzenesulfonyl-4-(bromoalkoxy)furoxan selected (**19a-c**) (1.82 mmol: **19a**, 635 mg; **19b**, 661 mg; **19c**, 687 mg) and K_2CO_3 (0.27 g, 1.99 mmol) in DMF (20 mL) was stirred at 22°C for 17 hours. The reaction mixture was poured onto ice-water (50 mL) to give a jelly or solid which was filtered and dried under vacuum. The product was dissolved in ethyl acetate (40 mL) and washed with water (3 x 30 mL). The product was separated by silica gel column chromatography using ethyl acetate-hexane at a suitable mixture ratio as eluant. If the product was a solid (**24-32**), it was recrystallized from ethyl acetate-diethyl ether. The percent yield, melting point, spectral and analytical data for products **24-38** are listed below.

3.1.2.4.1 5-(2-[3-(Benzenesulfonyl)furoxan-4-yloxy]ethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-pyridinyl)-5-pyridinecarboxylate (24)

Yield: 56% (solid); mp 148-149°C; IR ($CHCl_3$): 3281 (NH), 1712, 1620 (CO_2), 1549, 1454 (NO_2), 1370, 1164 (SO_2) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.32 (s, 3H, C-2 *Me*), 2.50 (s, 3H, C-6 *Me*), 4.47-4.51 (m, 2H, C*H*$_2$CH$_2$O), 4.55-4.59 (m, 2H, CH$_2$C*H*$_2$O), 5.60 (s, 1 H, H-4), 7.20 (d, $J_{4,5}$ = 7.3 Hz of d, $J_{5,6}$= 4.6 Hz, 1H, pyridinyl H-5), 7.69-7.58 (m, 4H, phenylsulfonyl H3-, H-4, H-5, pyridinyl H-3), 7.75 (d, $J_{3,4}$ = 7.3 Hz, of d, $J_{4,5}$ = 7.3 Hz, 1 H, pyridinyl H-4), 8.07 (d, *J* = 7.9 Hz, 2H, phenylsulfonyl H-2, H-6) 8.79 (d, $J_{5,6}$ = 4.6 Hz, 1H, pyridinyl H-6), 8.80 (s, 1H, N*H*). *Anal.* Calcd. for $C_{23}H_{21}N_5O_9S \bullet 1/2H_2O$: C 50.00, H 4.01, N 12.67. Found: C 49.95, H 3.74, N 12.51.

3.1.2.4.2 5-(3-[3-(Benzenesulfonyl)furoxan-4-yloxy]propyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-pyridinyl)-5-pyridinecarboxylate (25)

Yield: 48% (solid); mp 84-85°C; IR ($CHCl_3$): 3288 (NH), 1699, 1620 (CO_2), 1553, 1458 (NO_2), 1372, 1164 (SO_2) CM^{-1}; ^1H NMR ($CDCL_3$): δ 2.13-2.21 (m, 2H, $CH_2CH_2CH_2O$), 2.31 (s, 3H, C-2 *Me*), 2.48 (s, 3H, C-6 *Me*), 4.21-4.30 (m, 2H, $CH_2CH_2CH_2O$), 4.35 (t, J = 6.4 Hz, 2H, $CH_2CH_2CH_2O$), 5.57 (s, 1 H, H-4), 7.18 (d, $J_{4,5}$ = 7.3 Hz of d, $J_{5,6}$ = 4.0 Hz, 1H, pyridinyl H-5), 7.51 (d, $J_{3,4}$ = 7.6Hz, 1H, pyridinyl H-3), 7.63 (m, 3H, phenylsulfonyl H-3, H-4, H-5), 7.77 (d, $J_{3,4}$ = 7.6 Hz, of d, $J_{4,5}$= 7.3 Hz, 1 H, pyridinyl H-4), 8.06 (d, J = 8.1 Hz, 2H, phenylsulfonyl H-2, H-6), 8.36 (br s, 1H, N*H*), 8.47 (d, $J_{5,6}$ = 4.6 Hz, 1 H, pyridinyl H-6). *Anal.* Calcd. for $C_{24}H_{23}N_5O_9S$: C 51.70, H 4.16, N 12.56. Found: C 51.98, H 4.33, N 4.33.

3.1.2.4.3 5-(4-[3-(Benzenesulfonyl)furoxan-4-yloxy]butyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-pyridinyl)-5-pyridinecarboxylate (26)

Yield: 48% (solid); mp 168-169°C; IR ($CHCl_3$): 3275 (NH), 1698, 1620 (CO_2), 1552, 1459 (NO_2), 1371, 1164 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.80-1.84 (m, 4H, $CH_2CH_2CH_2CH_2O$), 2.30 (s, 3H, C-2 *Me*), 2.48 (s, 3H, C-6 *Me*), 4.11-4.16 (m, 2H, $CH_2CH_2CH_2CH_2O$), 4.39-4.43 (m, 2H, $CH_2CH_2CH_2CH_2O$), 5.60 (s, 1 H, H-4), 7.22 (d, $J_{4,5}$ = 7.6 Hz of d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-5), 7.51 (d, $J_{3,4}$ = 7.6 Hz, 1H, pyridinyl H-3), 7.58 (d, $J_{3,4}$ = 7.6 Hz of d, $J_{5,6}$ = 4.9 Hz, 1H, pyridinyl H-5), 7.62 (d, J = 7.6 Hz of d, J = 7.6 Hz, 2H, phenylsulfonyl H-3, H-5), 7.70 (t, J = 7.6 Hz, 1H, phenylsulfonyl H-4), 7.76 (d, $J_{3,4}$ = 7.6 Hz, of d, $J_{4,5}$ = 7.6 Hz, 1H, pyridinyl H-4), 8.06 (d, J = 7.9 Hz, 2H, phenylsulfonyl H-2, H-6), 8.48 (d, $J_{5,6}$ = 4.9 Hz, 1 H, pyridinyl H-6), 8.90 (br s, 1H, N*H*). *Anal.* Calcd. for $C_{25}H_{25}N_5O_9S$: C 52.54, H 4.41, N 12.25. Found: C 52.49, H 4.31, N 11.87.

3.1.2.4.4 5-(2-[3-(Benzenesulfonyl)furoxan-4-yloxy]ethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(3-pyridinyl)-5-pyridinecarboxylate (27)

Yield: 56% (solid); mp 148-149°C; IR ($CHCl_3$): 3281 (NH), 1710, 1610 (CO_2), 1553, 1442 (NO_2), 1370, 1164 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 2.32 (s, 3H, C-2 *Me*), 2.50 (s, 3H, C-6 *Me*), 4.47-4.51 (m, 2H, CH_2CH_2O), 4.55-4.59 (m, 2H,

CH_2CH_2O), 5.60 (s, 1H, H-4), 7.60-7.68 (m, 3H, pyridinyl H-4, phenylsulfonyl H-3, H-5), 7.78 (t, J = 7.6 Hz, 1H, phenylsulfonyl H-4), 8.07 (d, J = 7.6 Hz, 2H, phenylsulfonyl H-2, H-6), 8.36 (d, $J_{5,6}$ = 4.6 Hz, 1H, pyridinyl H-6), 8.55 (s, 1H, pyridinyl H-2), 8.80 (s, 1H, N*H*). *Anal.* Calcd. for $C_{23}H_{21}N_5O_9S \bullet 1/4H_2O$: C 50.41, H 4.03, N 12.77. Found: C 50.25, H 3.84, N 12.73.

3.1.2.4.5 5-(3-[3-(Benzenesulfonyl)furoxan-4-yloxy]propyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(3-pyridinyl)-5-pyridinecarboxylate (28)

Yield: 48% (solid); mp 84-85°C; IR ($CHCl_3$): 3295 (NH), 1709, 1612 (CO_2), 1543, 1448 (NO_2), 1367, 1169 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 1.83 (quintet, J = 6.4 Hz, 2H, $CH_2CH_2CH_2O$), 2.31 (s, 3H, C-2 *Me*), 2.48 (s, 3H, C-6 *Me*), 3.84-4.02 (m, 4H, $CH_2CH_2CH_2O$), 5.37 (s, 1H, H-4), 7.18 (d, $J_{4,5}$ = 7.3 Hz of d, $J_{5,6}$ = 4.0 Hz, 1H, pyridinyl H-5), 7.60-7.72 (m, pyridinyl H-4, phenylsulfonyl H-3, H-5), 7.77 (t, J = 7.6 Hz, 1H, phenylsulfonyl H-4), 8.06 (d, J = 7.6 Hz, 2H, phenylsulfonyl H-2, H-6), 8.36 (d, $J_{5,6}$ = 4.0 Hz, 1H, pyridinyl H-6), 8.54 (s, 1H, pyridinyl H-2), 9.02 (s, 1H, N*H*). *Anal.* Calcd. for $C_{24}H_{23}N_5O_9S$: C 51.70, H 4.16, N 12.56. Found: C 51.98, H 4.33, N 12.25.

3.1.2.4.6 5-(4-[3-(Benzenesulfonyl)furoxan-4-yloxy]butyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(3-pyridinyl)-5-pyridinecarboxylate (29)

Yield: 54% (solid); mp 160-161°C; IR ($CHCl_3$): 3288 (NH), 1704, 1617 (CO_2), 1550, 1434 (NO_2), 1373, 1163 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 1.70-1.88 (m, 4H, $CH_2CH_2CH_2CH_2O$), 2.39 (s, 3H, C-2 *Me*), 2.54 (s, 3H, C-6 *Me*), 4.04-4.20 (m, 2H, $CH_2CH_2CH_2CH_2O$), 4.30-4.42 (m, 2H, $CH_2CH_2CH_2CH_2O$), 5.37 (s, 1H, H-4), 7.18 (d, $J_{4,5}$ = 7.6 Hz of d, $J_{5,6}$ = 4.6 Hz, 1H, pyridinyl H-5), 7.54-7.68 (m, 3H, pyridinyl H-4, phenylsulfonyl H-3, H-5), 7.74 (t, J = 7.6 Hz, 1H, phenylsulfonyl H-4), 8.06 (d, J = 7.6 Hz, 2H, phenylsulfonyl H-2, H-6), 8.38 (d, $J_{5,6}$ = 4.6 Hz, 1H, pyridinyl H-6), 8.56 (s, 1H, pyridinyl H-2), 8.79 (br s, 1H, N*H*). *Anal.* Calcd. for $C_{25}H_{25}N_5O_9S$: C 52.54, H 4.41, N 12.25. Found: C 52.29, H 4.32, N 12.13.

3.1.2.4.7 5-(2-[3-(Benzenesulfonyl)furoxan-4-yloxy]ethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(4-pyridinyl)-5-pyridinecarboxylate (30)

Yield: 56% (solid); mp 109-110°C; IR ($CHCl_3$): 3212 (NH), 1710, 1618 (CO_2), 1551, 1450 (NO_2), 1370, 1163 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.19 (s, 3H, C-2 *Me*), 2.35 (s, 3H, C-6 *Me*), 4.24-4.29 (m, 2H, $COOCH_2CH_2O$), 4.30-4.36 (m, 2H, CH_2CH_2O), 5.16 (s, 1 H, H-4), 7.04-7.06 (m, 2H, pyridinyl H-3, H-5), 7.42 (t, J = 7.9 Hz, 2H, phenylsulfonyl H-3, H-5), 7.56 (t, J = 7.9 Hz, 1H, phenylsulfonyl H-4), 7.82 (d, J = 7.9 Hz, 2H, phenylsulfonyl H-2, H-6) 8.18-8.20 (m, 2H, pyridinyl H-2, H-6), 9.0 (s, 1H, N*H*); ^{13}C NMR ($CDCl_3$ + DMSO-d_6): δ 18.01 (DHP C-2 *Me*), 19.61 (DHP C-6 *Me*), 40.04 (DHP *C*-4), 60.82 ($CO_2CH_2CH_2$), 69.07 ($CO_2CH_2CH_2$), 104.29 (DHP *C*-3), 110.84 (furoxan C-3), 122.84 (pyridinyl *C*-3, *C*-5), 125.52 (DHP *C*-5), 128.02 (phenylsulfonyl *C*-3, *C*-5), 129.52 (phenylsulfonyl *C*-2, *C*-6), 135.59 (phenylsulfonyl *C*-4), 137.31 (phenylsulfonyl *C*-1), 145.86 (DHP *C*-2), 147.40 (DHP *C*-6), 149.06 (pyridinyl C-2, C-6), 152.93 (pyridinyl C-4), 158.26 (furoxan *C*-4), 165.31 (*C*O_2). *Anal.* Calcd. for $C_{23}H_{21}N_5O_9S$: C 50.83, H 3.89, N 12.89. Found: C 50.93, H 3.90, N 12.53.

3.1.2.4.8 5-(3-[3-(Benzenesulfonyl)furoxan-4-yloxy]propyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(4-pyridinyl)-5-pyridinecarboxylate (31)

Yield: 48% (solid); mp 84-85°C; IR ($CHCl_3$): 3311 (NH), 1703, 1617 (CO_2), 1554, 1459 (NO_2), 1373, 1164 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.18 (quintet, J = 6.1 Hz, 2H, $CH_2CH_2CH_2O$), 2.41 (s, 3H, C-2 *Me*), 2.54 (s, 3H, C-6 *Me*), 4.22-4.34 (m, 4H, $CH_2CH_2CH_2O$), 5.40 (s, 1 H, H-4), 6.64 (br s, 1H, N*H*), 7.25 (d, $J_{2,3} = J_{5,6}$ = 6.1 Hz, 2H, pyridinyl H-3, H-5), 7.62 (d, J = 7.3 Hz of d, J = 7.3 Hz, 2H, phenylsulfonyl H-3, H-5), 7.75 (t, J = 7.3 Hz, 1H, phenylsulfonyl H-4), 8.04 (d, J = 7.3 Hz, 2H, phenylsulfonyl H-2, H-6), 8.46 (d, $J_{2,3} = J_{5,6}$ = 6.1 Hz, 2H, pyridinyl H-2, H-6). *Anal.* Calcd. for $C_{24}H_{23}N_5O_9S$: C 51.70, H 4.16, N 12.56. Found: C 51.99, H 4.23, N 12.39.

3.1.2.4.9 5-(4-[3-(Benzenesulfonyl)furoxan-4-yloxy]butyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(4-pyridinyl)-5-pyridinecarboxylate (32)

Yield: 52% (solid); mp 78-80°C; IR ($CHCl_3$): 3313 (NH), 1704, 1617 (CO_2), 1553, 1461 (NO_2), 1371, 1162 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.68-1.72 and 1.76-1.80 (two m,

2H each, $CH_2CH_2CH_2CH_2O$), 2.43 (s, 3H, C-2 *Me*), 2.55 (s, 3H, C-6 *Me*), 4.10-4.17 (m, 2H, $CH_2CH_2CH_2CH_2O$), 4.36-4.43 (m, 2H, $CH_2CH_2CH_2CH_2O$), 5.43 (s, 1 H, H-4), 6.61 (br s, 1H, N*H*), 7.28 (d, $J_{2,3} = J_{5,6}$ = 4.6 Hz, 2H, pyridinyl H-3, H-5), 7.62 (d, *J* = 7.3 Hz of d, *J* = 7.3 Hz, 2H, phenylsulfonyl H-3, H-5), 7.77 (t, *J* = 7.3 Hz, 1H, phenylsulfonyl H-4), 8.06 (d, *J* = 7.3 Hz, 2H, phenylsulfonyl H-2, H-6), 8.49 (d, $J_{2,3} = J_{5,6}$ = 4.6 Hz, 2H, pyridinyl H-2, H-6). *Anal.* Calcd. for $C_{25}H_{25}N_5O_9S$: C 52.54, H 4.41, N 12.25. Found: C 52.69, H 4.20, N 11.87.

3.1.2.4.10 5-(2-[3-(Benzenesulfonyl)furoxan-4-yloxy]ethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-5-pyridinecarboxylate (33)

Yield: 57% (jelly); IR ($CHCl_3$): 3330 (NH), 1713, 1618 (CO_2), 1553, 1440 (NO_2), 1370, 1158 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.4 (s, 3H, C-2 *Me*), 2.52 (s, 3H, C-6 *Me*), 4.39-4.50 (m, 2H, CH_2CH_2O), 4.52-4.55 (m, 2H, CH_2CH_2O), 5.94 (s, 1H, N*H*), 5.98 (s, 1 H, H-4), 7.23-7.28 (m, 1H, trifluoromethylphenyl H-4), 7.40-7.50 (m, 3H, trifluoromethylphenyl H-3, H-5, H-6), 7.61 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-3, H-5), 7.72 (d, *J* = 7.6 Hz, of d, *J* = 7.6 Hz, 1H, phenylsufonyl H-4), 8.05 (d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-2, H-6). *Anal.* Calcd. for $C_{25}H_{21}F_3N_4O_9S$: C 49.20, H 3.50, N 9.20. Found: C 49.30, H 3.41, N 8.81.

3.1.2.4.11 5-(3-[3-(Benzenesulfonyl)furoxan-4-yloxy]propyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-5-pyridinecarboxylate (34)

Yield: 48% (jelly); IR ($CHCl_3$): 3332 (NH), 1705, 1620 (CO_2), 1553, 1475 (NO_2), 1369, 1158 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.05-2.18 (m, 2H, $CH_2CH_2CH_2$), 2.35 (s, 3H, C-2 *Me*), 2.50 (s, 3H, C-6 *Me*), 4.16-4.23 (m, 2H, $CH_2CH_2CH_2O$), 4.23-4.31 (m, 2H, $CH_2CH_2CH_2O$), 5.94 (s, 1 H, H-4), 6.02 (s, 1H, N*H*), 7.28-7.31 (m, 1H, trifluoromethylphenyl H-4), 7.40-7.49 (m, 2H, trifluoromethylphenyl H-5, H-6), 7.51 (d, *J* = 7.6 Hz, 1H, trifluoromethylphenyl H-3), 7.63 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-3, H-5), 7.76 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 1H, phenylsulfonyl H-4), 8.06 (d, *J* = 7.6 Hz, 2H, phenylsuflonyl H-2, H-6). *Anal.* Calcd. for $C_{26}H_{23}F_3N_4O_9S$: C 50.00, H 3.71, N 8.97. Found: C 49.98, H 3.68, N 8.84.

3.1.2.4.12 5-(4-[3-(Benzenesulfonyl)furoxan-4-yloxy]butyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-5-pyridinecarboxylate (35)

Yield: 50% (jelly); IR ($CHCl_3$): 3333 (NH), 1717, 1617 (CO_2), 1553, 1456 (NO_2), 1370, 1155 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.61-1.66 (m, 4H, $CH_2CH_2CH_2CH_2O$), 2.28 (s, 3H, C-2 *Me*), 2.43 (s, 3H, C-6 *Me*), 3.49-3.94 and 4.0-4.15 (two m, 1H each, $CH_2CH_2CH_2CH_2O$), 4.26-4.28 (m, 2H, $CH_2CH_2CH_2CH_2O$), 5.88 (s, 1 H, H-4), 5.90 (s, 1H, N*H*), 7.18-7.26 (m, 1H, trifluoromethylphenyl H-4), 7.34-7.40 (m, 2H, trifluoromethylphenyl H-5, H-6), 7.51 (d, *J* =7.6 Hz, 1H, trifluoromethylphenyl H-3), 7.55 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-3, H-5), 7.69 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 1H, phenylsulfonyl H-4), 7.97 (d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-2, H-6). *Anal.* Calcd. for $C_{27}H_{25}F_3N_4O_9S$: C 50.78, H 3.95, N 8.97. Found: C 50.97, H 3.94, N 8.62.

3.1.2.4.13 5-(2-[3-(Benzenesulfonyl)furoxan-4-yloxy]ethyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)-5-pyridinecarboxylate (36)

Yield: 63% (jelly); IR ($CHCl_3$): 3327 (NH), 1705, 1622 (CO_2), 1551, 1469 (NO_2), 1360, 1164 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.40 (s, 3H, C-2 *Me*), 2.57 (s, 3H, C-6 *Me*), 4.42-4.52 (m, 2H, $COOCH_2CH_2O$), 4.53-4.62 (m, 2H, CH_2CH_2O), 5.84 (s, 1 H, H-4), 6.32 (br s, 1H, N*H*), 7.34 (d, $J_{5,6}$ = 6.7 Hz of d, $J_{6,7}$ = 8.8 Hz, 1H, benzofurazanyl H-6), 7.51 (d, $J_{5,6}$ = 6.7 Hz, 1H, benzofurazanyl H-5), 7.61 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-3, H-5), 7.67 (d, $J_{6,7}$ = 8.8 Hz, 1H, benzofurazanyl H-7), 7.74 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 1H, phenylsulfonyl H-4), 8.06 (d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-2, H-6). *Anal.* Calcd. for $C_{24}H_{20}N_6O_{10}S$: C 49.32, H 3.45, N 14.38. Found: C 49.56, H 3.45, N 14.03.

3.1.2.4.14 5-(3-[3-(Benzenesulfonyl)furoxan-4-yloxy]propyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)-5-pyridinecarboxylate (37)

Yield: 48% (jelly); IR ($CHCl_3$): 3328 (NH), 1714, 1620 (CO_2), 1553, 1460 (NO_2), 1374, 1157 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.13-2.21 (m, 2H, $CO_2CH_2CH_2CH_2O$), 2.39 (s, 3H, C-2 *Me*), 2.58 (s, 3H, C-6 *Me*), 4.25 (t, *J* = 6.2 Hz, 2H, $CO_2CH_2CH_2CH_2O$), 4.38 (t, *J* = 6.2 Hz, 2H, $CO_2CH_2CH_2CH_2O$), 5.82 (s, 1H, H-4), 6.26 (br s, 1H, N*H*), 7.32 (d, $J_{5,6}$ = 6.4 Hz of d, $J_{6,7}$ = 8.8 Hz, 1H, benzofurazanyl H-6), 7.42 (d, $J_{5,6}$ = 6.4 Hz, 1H, benzofurazanyl H-5), 7.62 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-3, H-5),

7.68 (d, $J_{6,7}$ = 8.8 Hz, 1H, benzofurazanyl H-7), 7.76 (t, *J* = 7.6 Hz, 1H, phenylsulfonyl H-4), 8.06 (d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-2, H-6). *Anal.* Calcd. for $C_{25}H_{22}N_6O_{10}S \bullet 1/2H_2O$: C 49.42, H 3.81, N 13.83. Found: C 49.47, H 3.74, N 13.07.

3.1.2.4.15 5-(4-[3-(Benzenesulfonyl)furoxan-4-yloxy]butyl) 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(benzofurazan-4-yl)-5-pyridinecarboxylate (38)

Yield: 28% (jelly); IR ($CHCl_3$): 3324 (NH), 1696, 1620 (CO_2), 1552, 1464 (NO_2), 1369, 1162 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.76-1.84 (m, 4H, $CH_2CH_2CH_2CH_2O$), 2.40 (s, 3H, C-2 *Me*), 2.56 (s, 3H, C-6 *Me*), 4.14 (t, *J* = 6.1 Hz, 2H, $CH_2CH_2CH_2CH_2O$), 4.42 (t, *J* = 6.1 Hz, 2H, $CH_2CH_2CH_2CH_2O$), 5.83 (s, 1 H, H-4), 6.31 (br s, 1H, N*H*), 7.37 (d, $J_{5,6}$ = 6.4 Hz of d, $J_{6,7}$ = 8.8 Hz, 1H, benzofurazanyl H-6), 7.47 (d, $J_{5,6}$ = 6.4 Hz, 1H, benzofurazanyl H-5), 7.63 (d, *J* = 7.6 Hz of d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-3, H-5), 7.68 (d, $J_{6,7}$ = 8.8 Hz, 1H, benzofurazanyl H-7), 7.76 (t, *J* = 7.6 Hz, 1H, phenylsulfonyl H-4), 8.06 (d, *J* = 7.6 Hz, 2H, phenylsulfonyl H-2, H-6). *Anal.* Calcd. for $C_{28}H_{24}N_6O_{10}S \bullet H_2O$: C 49.52, H 4.16, N 13.33. Found: C 49.30, H 3.83, N 12.40.

3.1.3 1,4-Dihydropyridine-(*N*-Nitrosoamine) Hybrids

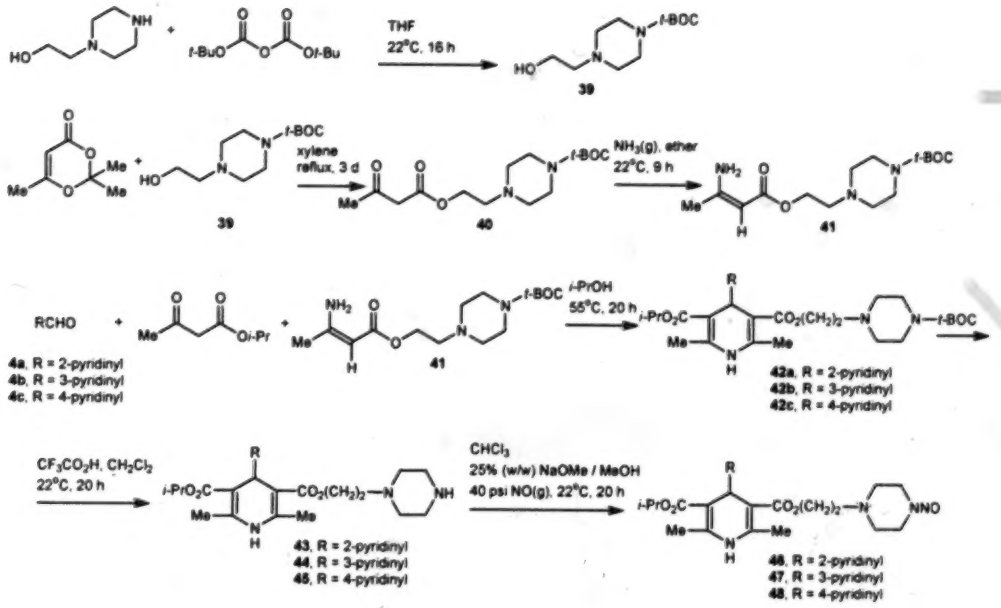


Figure 3.1.3: Synthesis of 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**46-48**)

3.1.3.1 1-(*t*-Butoxycarbonyl)-4-(2-hydroxyethyl)piperazine (39)

t-Butyl pyrocarbonate (43.65 g, 200 mmol) in THF (50 mL) was added drop-wise to 1-(2-hydroxyethyl)piperazine (26.04 g, 200 mmol) in THF (50 mL) at 0°C with stirring. This mixture was stirred at 22°C for 16 hours. Removal of the solvent *in vacuo* gave an oily residue (46.04 g) that solidified in hexane at -5°C over 4 hours. The spectral data matched those reported by *Radau et al.* (2003).

Yield: quantitative (wax; *Radau et al.* [2003] reported this compound as an oil); mp: 34°C; ^{1}H NMR ($CDCl_3$): δ 1.47 (s, 9H, *t*-BOC Me's), 2.46 (t, *J* = 5.0 Hz, 4H, piperazinyl H-3 and H-5), 2.56 (t, *J* = 5.4 Hz, 2H, CH_2N), 3.44 (t, *J* = 5.0 Hz, 4H, piperazinyl H-2 and H-6), 3.63 (t, *J* = 5.4 Hz, 2H, CH_2O).

3.1.3.2 2-(4-[*t*-Butoxycarbonyl]piperazinyl)ethyl Acetoacetate (40)

2-(4-[*t*-Butoxycarbonyl]piperazinyl)ethyl acetoacetate (**40**) was prepared by modification of the general method described in Section 3.1.1.2. A solution of 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (9.82 g, 69 mmol) and 1-(*t*-butoxycarbonyl)-4-(2-hydroxyethyl)piperazine (**39**) (15.90 g, 69 mmol) in xylene (50 mL) was refluxed for 3 days, after which the solvent was removed *in vacuo*. The residue was dissolved in diethyl ether (100 mL), washed with water (3 × 75 mL), dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*.

Yield: 61% (oil); ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 9H, *t*-BOC Me's), 2.29 (s, 3H, acetoacetate Me), 2.44 (t, *J* = 5.0 Hz, 4H, piperazinyl H-2 and H-6), 2.65 (t, *J* = 5.8 Hz, 2H, CH_2N), 3.42 (t, *J* = 5.0 Hz; 4H, piperazinyl H-3 and H-5), 3.48 (s, 2H, acetoacetate CH_2), 4.28 (t, *J* = 5.8 Hz, 2H, CH_2O).

3.1.3.3 2-(4-[*t*-Butoxycarbonyl]piperazinyl)ethyl 3-Aminocrotonate (41)

2-(4-[*t*-Butoxycarbonyl]piperazinyl)ethyl 3-aminocrotonate (**41**) was prepared by according to the general method described by Koo (1953). Optimal yield and ease of purification were attained when the reaction proceeded for 9 hours at 22°C.

Yield: 97% (oil); ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H, *t*-BOC Me's), 1.91 (s, 3H, aminocrotonate *Me*), 2.46 (t, *J* = 5.0 Hz, 4H, piperazinyl H-2 and H-6), 2.65 (t, *J* = 5.9 Hz, 2H, CH_2N), 3.43 (t, *J* = 5.0 Hz, 4H, piperazinyl H-3 and H-5), 4.19 (t, *J* = 5.9 Hz, 2H, CH_2O), 4.54 (s, 1H, aminocrotonate CH).

3.1.3.4 General Method for the Synthesis of 3-Isopropyl 5-(2-[4-(*t*-Butoxycarbonyl)piperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (42a-c)

3-Isopropyl 5-(2-[4-(*t*-Butoxycarbonyl)piperazinyl]ethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**42a-c**) were prepared by modification of the general method described in Section 3.1.1.4. A solution of pyridinecarboxaldehyde (**4a-c**) (2.36 g, 22 mmol) and isopropyl acetoacetate (3.17 g, 22 mmol) in isopropanol (10 mL) was stirred for 10 minutes at 55°C. 2-(4-[*t*-Butoxycarbonyl]piperazinyl)ethyl 3-aminocrotonate (**41**) (6.27 g, 20 mmol) was added and the reaction was allowed to proceed for 20 hours, after which the solvent was removed *in vacuo*. The residue was dissolved in diethyl ether (100 mL), washed with water (3 × 75 mL), dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*. A powder was

crystallized from a mixture of diethyl ether / petroleum ether at 5°C for 20 hours. The powder was then recrystallized from diethyl ether. The percent yield, melting point, and spectral data for products **42a-c** are listed below.

3.1.3.4.1 3-Isopropyl 5-(2-[4-(*t*-Butoxycarbonyl)piperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (42a)

Yield: 31% (solid); mp: 145°C; ^{1}H NMR ($CDCl_3$): δ 1.10 and 1.22 (two d, J = 6.2 Hz, 3H each, isopropyl Me's), 1.47 (s, 9H, *t*-BOC Me's), 2.26 and 2.36 (two s, 3H each, C-2 and C-6 Me's), 2.38 (t, J = 5.0 Hz, 4H, piperazinyl H-2 and H-6), 2.58 (t, J = 5.8 Hz, 2H, CH_2N), 3.38 (t, J = 5.0 Hz, 4H, piperazinyl H-3 and H-5), 4.16 (t, J = 5.8 Hz, 2H, CH_2O), 4.95 (septet, J = 6.2 Hz, 1H, isopropyl CH), 5.17 (s, 1H, H-4), 7.13 (ddd, $J_{4,5}$ = 8.1, $J_{5,6}$ = 4.8, $J_{3,5}$ = 0.5 Hz, 1H, pyridinyl H-5), 7.41 (d, $J_{3,4}$ = 8.3 Hz, 1H, pyridinyl H-3), 7.56 (ddd, $J_{3,4}$ = 8.3, $J_{4,5}$ = 8.1, $J_{4,6}$ = 0.9 Hz, 1H, pyridinyl H-4), 8.17 (broad s, 1H, NH), 8.49 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6). *Anal.* Calcd. for $C_{28}H_{40}N_4O_6$: C 63.62, H 7.63, N 10.60. Found: C 63.19, H 7.81, N 10.47. *m/z* (ES+) (Figure 3.1.3.4.1).

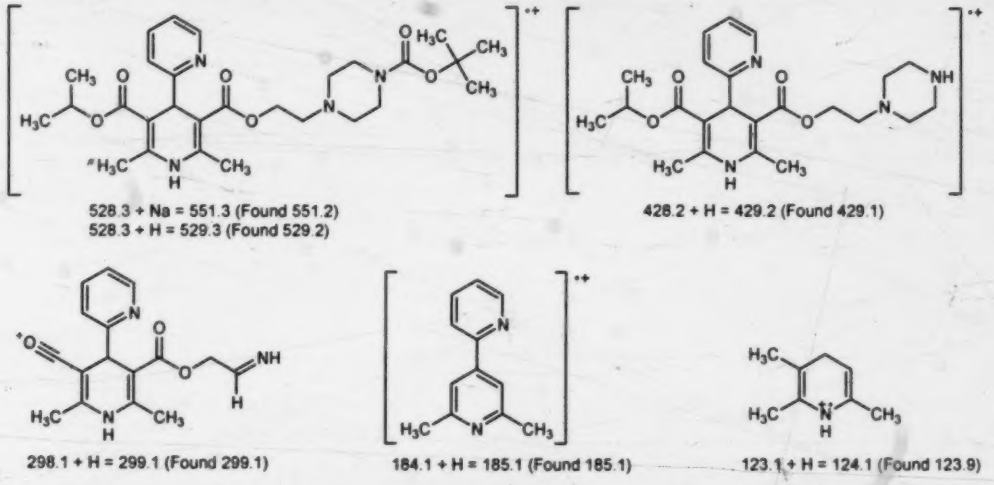


Figure 3.1.3.4.1: Fragmentation Pattern of 3-Isopropyl 5-(2-[4-(*t*-Butoxycarbonyl)piperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**42a**)

3.1.3.4.2 3-Isopropyl 5-(2-[4-(*t*-Butoxycarbonyl)piperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (42b)

Yield: 59% (solid); mp: 132-134°C; ^{1}H NMR ($CDCl_3$): δ 1.11 and 1.25 (two d, J = 6.4 Hz, 3H each, isopropyl Me's), 1.46 (s, 9H, *t*-BOC Me's), 2.35 and 2.36 (two s, 3H each, C-2 and C-6 Me's), 2.33 – 2.43 (m, 4H, piperazinyl H-2 and H-6), 2.58 and 2.59 (two dd, J_{gem} = 11.6, J_{vic} = 4.9 Hz, 1H each, CH_2N), 3.39 (t, J = 5.0 Hz, 4H, piperazinyl H-3 and H-5), 4.16 and 4.17 (two dd, J_{gem} = 12.1, J_{vic} = 4.9 Hz, 1H each, CH_aH_bO), 4.96 (septet, J = 6.4 Hz, 1H, isopropyl CH), 4.97 (s, 1H, H-4), 5.9 (sharp s, 1H, NH), 7.15 (dd, $J_{4,5}$ = 8.7, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-5), 7.61 (dd, $J_{4,5}$ = 8.7, $J_{4,6}$ = 2.6 Hz, 1H, pyridinyl H-4), 8.37 (d, $J_{4,6}$ = 2.6, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-6), 8.54 (d, $J_{2,4}$ = 1.9 Hz, 1H, pyridinyl H-2). *Anal.* Calcd. for $C_{28}H_{40}N_4O_6 \bullet 1/10H_2O$: C 63.40, H 7.64, N 10.56. Found: C 63.19, H 7.96, N 10.38.

3.1.3.4.3 3-Isopropyl 5-(2-[4-(*t*-Butoxycarbonyl)piperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (42c)

Yield: 58% (solid); mp: 62-63°C; ^{1}H NMR ($CDCl_3$): δ 1.12 and 1.24 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 1.46 (s, 9H, *t*-BOC Me's), 2.34 and 2.35 (two s, 3H each, C-2 and C-6 Me's), 2.33 – 2.43 (m, 4H, piperazinyl H-2 and H-6), 2.58 and 2.59 (two dd, J_{gem} = 11.6, J_{vic} = 4.9 Hz, 1H each, CH_2N), 3.37 (t, J = 4.6 Hz, 4H, piperazinyl H-3 and H-5), 4.16 and 4.17 (two dd, J_{gem} = 12.1, J_{vic} = 4.9 Hz, 1H each, CH_aH_bO), 4.97 (septet, J = 6.1 Hz, 1H, isopropyl CH), 4.99 (s, 1H, H-4), 5.90 (sharp s, 1H, NH), 7.21 (d, $J_{2,3}$ = $J_{5,6}$ = 6.0 Hz, 2H, pyridinyl H-3 and H-5), 8.43 (d, $J_{2,3}$ = $J_{5,6}$ = 6.0 Hz, 2H, pyridinyl H-2 and H-6). *Anal.* Calcd. for $C_{28}H_{40}N_4O_6 \bullet 1/10H_2O$: C 63.40, H 7.64, N 10.56. Found: C 63.01, H 7.78, N 10.22.

3.1.3.5 General Method for the Synthesis of 3-Isopropyl 5-(2-Piperazinylethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (43-45)

Trifluoroacetic acid (19.3 mL, 250 mmol) was added drop-wise to a 3-isopropyl 5-(2-[4-(*t*-butoxycarbonyl)piperazinyl]ethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylate (**42a-c**) (2.65 g, 5 mmol) in dichloromethane (20 mL) and stirred at 22°C for 20 hours. The mixture was adjusted to pH 8 with a saturated solution of $NaHCO_3$(aq), extracted with dichloromethane (4 × 100 mL), washed with water (4 × 75

mL), dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*. The residue was dissolved in a minimal amount of ethyl acetate to which diethyl ether was added drop-wise until oiling-out occurred. The oiling-out portion was discarded. Removal of the solvent *in vacuo* gave a waxy residue that was further dried *in vacuo* while using diethyl ether as a co-solvating agent. The percent yield, melting point, spectral and analytical data for products **43-45** are listed below.

3.1.3.5.1 3-Isopropyl 5-(2-Piperazinylethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (43)

Yield: 34% (solid); mp: 65-66°C; IR ($CHCl_3$): 3436 (NH), 1690 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.09 and 1.21 (two d, J = 6.3 Hz, 3H each, isopropyl Me's), 2.24 and 2.25 (two s, 3H each, C-2 and C-6 Me's), 2.37 – 2.47 (m, 4H, piperazinyl H-2 and H-6), 2.56 (t, J = 6.0 Hz, 2H, CH_2N), 2.85 (t, J = 4.7 Hz, 4H, piperazinyl H-3 and H-5), 4.15 (t, J = 6.0 Hz, 2H, CH_2O), 4.95 (septet, J = 6.3 Hz, 1H, isopropyl CH), 5.17 (s, 1H, H-4), 7.14 (ddd, $J_{4,5}$ = 8.1, $J_{5,6}$ = 4.8, $J_{3,5}$ = 0.5 Hz, 1H, pyridinyl H-5), 7.44 (d, $J_{3,4}$ = 8.3 Hz, 1H, pyridinyl H-3), 7.57 (ddd, $J_{3,4}$ = 8.3, $J_{4,5}$ = 8.1, $J_{4,6}$ = 0.9 Hz, 1H, pyridinyl H-4), 8.41 (sharp s, 1H, DHP NH), 8.48 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6), the piperazinyl NH was not observed. *Anal.* Calcd. for $C_{23}H_{32}N_4O_4 \bullet 1/2H_2O$: C 63.14, H 7.60, N 12.81. Found: C 63.51, H 7.46, N 12.37.

3.1.3.5.2 3-Isopropyl 5-(2-Piperazinylethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (44)

Yield: 45% (solid); mp: 64-66°C; IR ($CHCl_3$): 3429 (NH), 1697 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.15 and 1.21 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 2.28 and 2.29 (two s, 3H each, C-2 and C-6 Me's), 2.27 – 2.37 (m, 4H, piperazinyl H-2 and H-6), 2.53 and 2.54 (two dd, J_{gem} = 12.0, J_{vic} = 4.9 Hz, 1H each, CH_2N), 2.81 (t, J = 4.8 Hz, 4H, piperazinyl H-3 and H-5), 4.11 and 4.12 (two dd, J_{gem} = 12.1, J_{vic} = 4.9 Hz, 1H each, CH_aH_bO), 4.91 (septet, J = 6.1 Hz, 1H, isopropyl CH), 4.94 (s, 1H, H-4), 7.12 (dd, $J_{4,5}$ = 8.7, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-5), 7.14 (broad s, 1H, DHP NH), 7.60 (dd, $J_{4,5}$ = 8.7, $J_{4,6}$ = 2.6 Hz, 1H, pyridinyl H-4), 8.31 (d, $J_{4,6}$ = 2.6, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-6), 8.49 (d, $J_{2,4}$ = 1.9 Hz, 1H, pyridinyl H-2), the piperazinyl NH was not observed. ^{13}C NMR ($CDCl_3$): δ 19.04 and 19.13 (C-2 and C-6), 21.78 and 22.07 (isopropyl Me), 37.65 (C-4),

46.00 (piperazinyl C-3 and C-5), 54.58 (piperazinyl C-2 and C-6), 57.19 (CH_2N), 61.06 (CH_2O), 67.00 (isopropyl CH), 102.52 and 103.31 (C-3 and C-5), 122.92 (pyridinyl C-5), 135.63 (pyridinyl C-4), 143.42 (pyridinyl C-3), 144.77 and 145.46 (C-2 and C-6), 146.84 (pyridinyl C-6), 149.35 (pyridinyl C-2), 166.53 and 166.99 (C=O). *Anal.* Calcd. for $C_{23}H_{32}N_4O_4$: C 64.46, H 7.53, N 13.07. Found: C 64.38; H 7.67 N 11.54.

3.1.3.5.3 3-Isopropyl 5-(2-Piperazinylethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (45)

Yield: 47% (solid); mp: 67-68°C; IR ($CHCl_3$): 3436 (NH), 1690 (C=O); ^{1}H NMR ($CDCl_3$): δ ^{1}H NMR ($CDCl_3$): δ 1.12 and 1.24 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 2.35 and 2.36 (two s, 3H each, C-2 and C-6 Me's), 2.35 – 2.45 (m, 4H, piperazinyl H-2 and H-6), 2.57 (two dd, J_{gem} = 12.0, J_{vic} = 4.9 Hz, 1H each, CH_2N), 2.85 (t, J = 4.7 Hz, 4H, piperazinyl H-3 and H-5), 4.16 - 4.17 (two dd, J_{gem} = 12.1, J_{vic} = 4.9 Hz, 1H each, CH_aH_bO), 4.97 (septet, J = 6.1 Hz, 1H, isopropyl CH), 5.00 (s, 1H, H-4), 5.75 (broad s, 1H, DHP NH), 7.23 (d, $J_{2,3} = J_{5,6}$ = 5.34 Hz, 2H, pyridinyl H-3 and H-5), 8.43 (d, $J_{2,3} = J_{5,6}$ = 5.34 Hz, 2H, pyridinyl H-2 and H-6), the piperazinyl NH was not observed. *Anal.* Calcd. for $C_{23}H_{32}N_4O_4$: C 64.46, H 7.53, N 13.07. Found: C 64.65, H 7.77, N 11.50.

3.1.3.6 General Method for the Synthesis of 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (46-48)

Twenty-five percent (w/w) sodium methoxide in methanol (320 mg, 1.5 mmol) was added drop-wise to a 3-isopropyl 5-(2-piperazinylethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylate (**43-45**) in chloroform (5 mL). The mixture was stirred at 22°C under NO(g) (40 psi) for 20 hours, after which the solvent was removed *in vacuo*. Dichloromethane (50 mL) was added and the insoluble solids were discarded. The solvent was removed *in vacuo* to give an oily residue. The residue was dissolved in a minimal amount of ethyl acetate to which diethyl ether was added drop-wise until oiling-out occurred. The oiling-out portion was discarded. Removal of the solvent *in vacuo* gave a waxy residue that was further dried *in vacuo* while using diethyl ether as a co-solvating agent. The residue was recrystallized from ethyl acetate / diethyl ether to give a powder. The percent yield, melting point, spectral and analytical data for products **46-48** are listed below.

3.1.3.6.1 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (46)

Yield: 19% (solid); mp: 143-145°C; IR ($CHCl_3$): 3436 (NH), 1690 (C=O), 1484 (N=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.11 and 1.22 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 2.26 and 2.28 (two s, 3H each, C-2 and C-6 Me's), 2.39 and 2.40 (two dd, J_{gem} = 10.0, J_{vic} = 4.6 Hz, 1H each, CH_2N), 2.63 and 2.64 (two dd, J_{gem} = unknown, J_{vic} = 5.2 Hz, 2H each, piperazinyl H-2 and H-6), 3.75 and 3.76 (two dd, J_{gem} = 11.9, J_{vic} = 4.6 Hz, 1H each, CH_aH_bO), 4.15 – 4.20 (four dd, J_{gem} = unknown, J_{vic} = 5.2 Hz, 1H each, piperazinyl H-3 and H-5), 4.96 (septet, J = 6.1 Hz, 1H, isopropyl CH), 5.17 (s, 1H, H-4), 7.13 (ddd, $J_{4,5}$ = 8.1, $J_{5,6}$ = 4.8, $J_{3,5}$ = 0.5 Hz, 1H, pyridinyl H-5), 7.39 (d, $J_{3,4}$ = 8.3 Hz, 1H, pyridinyl H-3), 7.57 (ddd, $J_{3,4}$ = 8.3, $J_{4,5}$ = 8.1, $J_{4,6}$ = 0.9 Hz, 1H, pyridinyl H-4), 8.01 (sharp s, 1H, NH), 8.50 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6). *Anal.* Calcd. for $C_{23}H_{31}N_5O_5$ (not within ±0.4%): C 60.38, H 6.83, N 15.31. Found: C 61.54, H 7.63, N 13.35. *m/z* (ES+) (Figure 3.1.3.6.1).

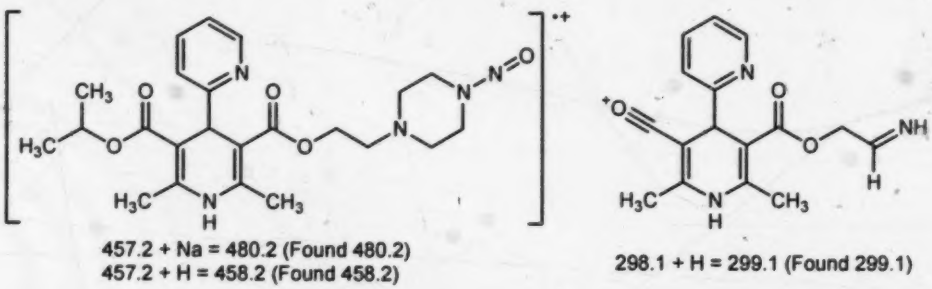


Figure 3.1.3.6.1: Fragmentation Pattern of 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**46**)

3.1.3.6.2 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (47)

Yield: 41% (solid); mp: 193-195°C; IR ($CHCl_3$): 3436 (NH), 1696 (C=O), 1486 (N=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.13 and 1.25 (two d, J = 6.2 Hz, 3H each, isopropyl Me's), 2.35 and 2.37 (two s, 3H each, C-2 and C-6 Me's), 2.40 (t, J = 5.5 Hz, 2H, CH_2N),

2.62 and 2.64 (two dd[14], J_{gem} = 10.7, J_{vic} = 5.3 Hz, 2H each, piperazinyl H-2 and H-6), 3.77 (t, J = 5.5 Hz, 2H, CH_2O), 4.13, 4.16, 4.21 and 4.26 (four dd[15], J_{gem} = 12.7, J_{vic} = 5.3 Hz, 1H each, piperazinyl H-3 and H-5), 4.97 (septet, J = 6.2 Hz, 1H, isopropyl CH), 4.98 (s, 1H, H-4), 5.98 (sharp s, 1H, NH), 7.16 (dd, $J_{4,5}$ = 8.7, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-5), 7.61 (dd, $J_{4,5}$ = 8.7, $J_{4,6}$ = 2.6 Hz, 1H, pyridinyl H-4), 8.37 (d, $J_{4,6}$ = 2.6, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-6), 8.56 (d, $J_{2,4}$ = 1.9 Hz, 1H, pyridinyl H-2). ^{13}C NMR ($CDCl_3$): δ 19.19 and 19.22 (C-2 and C-6), 21.22 and 22.11 (isopropyl Me), 37.65 (C-4), 39.54 and 49.67 (piperazinyl C-3 and C-5), 51.65 and 53.09 (piperazinyl C-2 and C-6), 56.18 (CH_2N), 60.61 (CH_2O), 67.04 (isopropyl CH), 102.22 and 103.43 (C-3 and C-5), 123.07 (pyridinyl C-5), 135.62 (pyridinyl C-4), 143.38 (pyridinyl C-3), 144.67 and 145.85 (C-2 and C-6), 146.91 (pyridinyl C-6), 149.33 (pyridinyl C-2), 166.51 and 166.88 (C=O). *Anal.* Calcd. for $C_{23}H_{31}N_5O_5$ (not within ±0.4%): C 60.38, H 6.83, N 15.31. Found: C 61.38, H 6.48, N 13.23. *m/z* (ES+) (Figure 3.1.3.6.2).

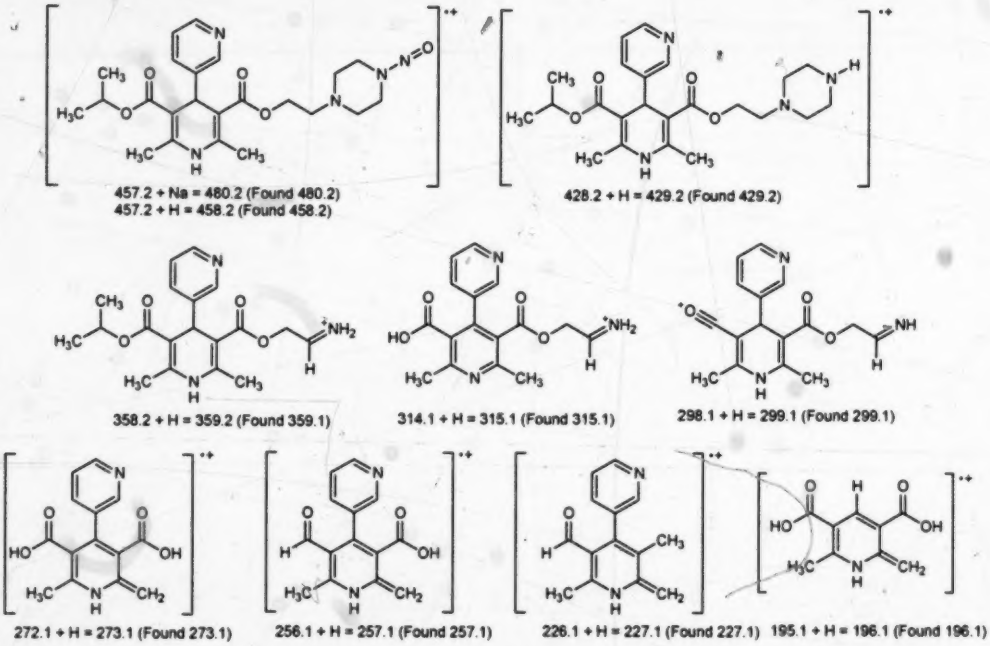


Figure 3.1.3.6.2: Fragmentation Pattern of 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (**47**)

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[14] Data acquired from a decoupling study by irradiating δ 4.1 – 4.3 (piperazinyl H-3 and H-5)

[15] Data acquired from a decoupling study by irradiating δ 2.6 – 2.7 (piperazinyl H-2 and H-6)

3.1.3.6.3 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (48)

Yield: 69% (solid); mp: 140-142°C; IR ($CHCl_3$): 3443 (NH), 1696 (C=O) , 1488 (N=O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.15 and 1.25 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 2.35 and 2.38 (two s, 3H each, C-2 and C-6 Me's), 2.40 and 2.41 (two dd, J_{gem} = 11.0, J_{vic} = 4.6 Hz, 1H each, CH_aH$_b$N), 2.37 (two dd, J_{gem} = unknown, J_{vic} = 5.6 Hz, 2H each, piperazinyl H-2 and H-6), 3.76 and 3.77 (two dd, J_{gem} = 11.9, J_{vic} = 4.6 Hz, 1H each, CH_aH$_b$O), 4.12 – 4.29 (m, 4H, piperazinyl H-3 and H-5), 4.97 (septet, J = 6.1 Hz, 1H, isopropyl CH), 5.00 (s, 1H, H-4), 5.84 (sharp s, 1H, NH), 7.23 (d, $J_{2,3}$ = $J_{5,6}$ = 6.0 Hz, 2H, pyridinyl H-3 and H-5), 8.45 (d, $J_{2,3}$ = $J_{5,6}$ = 6.0 Hz, 2H, pyridinyl H-2 and H-6). ^{13}C NMR ($CDCl_3$) δ 19.24 (C-2 and C-6), 21.82 and 22.10 (isopropyl Me), 39.31 (C-4), 39.52 and 49.66 (piperazinyl C-3 and C-5), 51.67 and 53.11 (piperazinyl C-2 and C-6), 56.17 (CH_2N), 60.74 (CH_2O), 67.26 (isopropyl CH), 101.50 and 102.79 (C-3 and C-5), 123.20 (pyridinyl C-3 and C-5), 144.92 and 146.16 (C-2 and C-6), 149.00 (pyridinyl C-2 and C-6), 156.15 (pyridinyl C-4), 166.51 and 166.81 (C=O). *m/z* (ES+) (Figure 3.1.3.6.3).

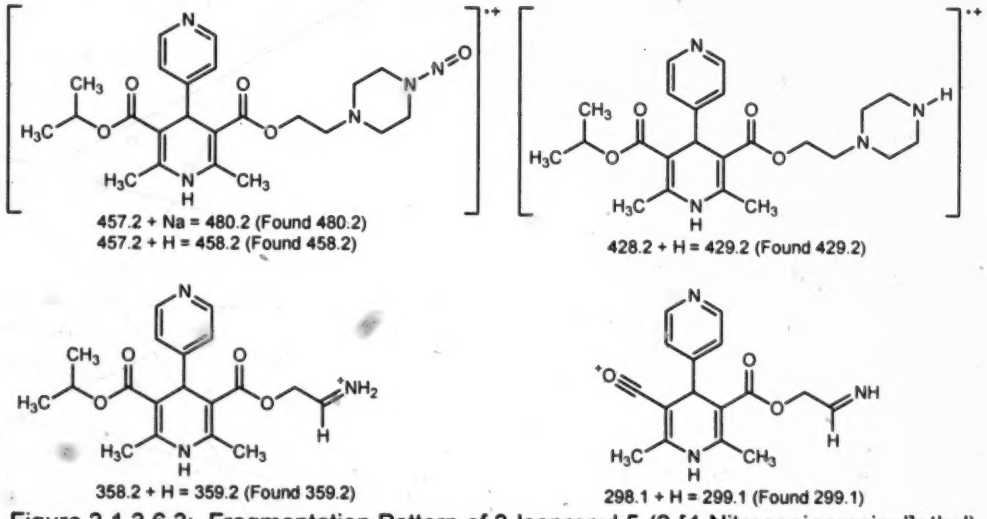


Figure 3.1.3.6.3: Fragmentation Pattern of 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (48)

3.1.4 1,4-Dihydropyridine-(N^1-Substituted Diazen-1-ium-1,2-diolate) Hybrids

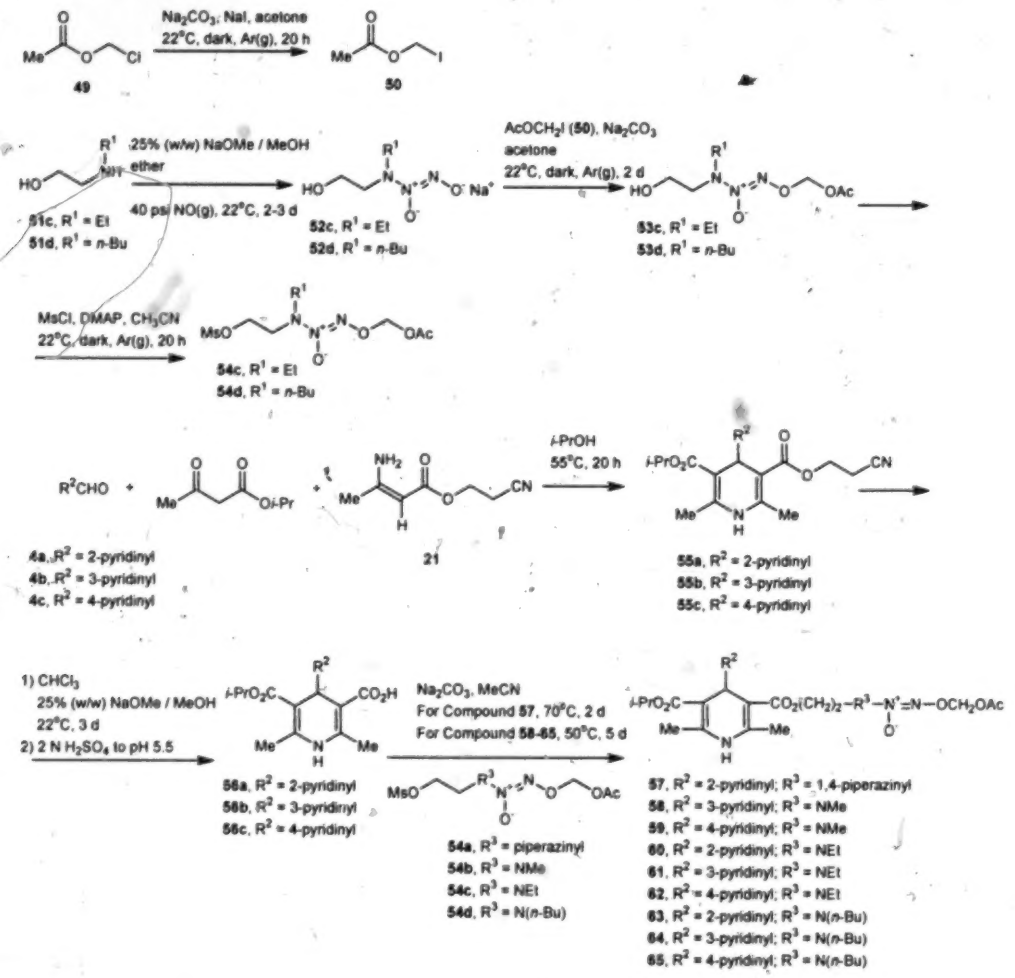

Na2CO3, NaI, acetone
22°C, dark, Ar(g), 20 h
49
50
25% (w/w) NaOMe / MeOH
ether
40 psi NO(g), 22°C, 2-3 d
51c, R1 = Et
51d, R1 = n-Bu
52c, R1 = Et
52d, R1 = n-Bu
AcOCH2I (50), Na2CO3
acetone
22°C, dark, Ar(g), 2 d
53c, R1 = Et
53d, R1 = n-Bu
MsCl, DMAP, CH3CN
22°C, dark, Ar(g), 20 h
54c, R1 = Et
54d, R1 = n-Bu
R2CHO
4a, R2 = 2-pyridinyl
4b, R2 = 3-pyridinyl
4c, R2 = 4-pyridinyl
21
i-PrOH
55°C, 20 h
55a, R2 = 2-pyridinyl
55b, R2 = 3-pyridinyl
55c, R2 = 4-pyridinyl
1) CHCl3
25% (w/w) NaOMe / MeOH
22°C, 3 d
2) 2 N H2SO4 to pH 5.5
56a, R2 = 2-pyridinyl
56b, R2 = 3-pyridinyl
56c, R2 = 4-pyridinyl
Na2CO3, MeCN
For Compound 57, 70°C, 2 d
For Compound 58-65, 50°C, 5 d
54a, R3 = piperazinyl
54b, R3 = NMe
54c, R3 = NEt
54d, R3 = N(n-Bu)
57, R2 = 2-pyridinyl; R3 = 1,4-piperazinyl
58, R2 = 3-pyridinyl; R3 = NMe
59, R2 = 4-pyridinyl; R3 = NMe
60, R2 = 2-pyridinyl; R3 = NEt
61, R2 = 3-pyridinyl; R3 = NEt
62, R2 = 4-pyridinyl; R3 = NEt
63, R2 = 2-pyridinyl; R3 = N(n-Bu)
64, R2 = 3-pyridinyl; R3 = N(n-Bu)
65, R2 = 4-pyridinyl; R3 = N(n-Bu)

Figure 3.1.4: Synthesis of 3-Isopropyl 5-(2-[(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)(*N*,*N*-dialkylamino or 4-piperazin-1-yl)]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**57-65**)

3.1.4.1 Iodomethyl Acetate (50)

Iodomethyl acetate (**50**) was prepared by modification of previously reported procedures [Velázquez & Knaus, 2004]. At 22°C in the dark and under an argon atmosphere, sodium carbonate (8.63 g, 81 mmol) then sodium iodide (10.71 g, 72 mmol)

were added a solution of choloromethyl acetate (**49**) (5.20 g, 48 mmol) in acetone (50 mL). After stirring for 20 hours, the insoluble inorganic salts were removed by filtering through Celite 545™, and the solvent was removed *in vacuo*. The oily residue was dissolved in ethyl acetate (100 mL), washed with sodium thiosulfate(aq) (2 N, 3 × 50 mL) then water (2 × 50 mL), dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*. Due to its highly unstable nature, the residue was immediately used for the preparation of O^2-acetoxymethyl 1-(*N*-[2-hydroxyethyl]-*N*-ethylamino)diazen-1-ium-1,2,-diolate (**53c**) and O^2-acetoxymethyl 1-(*N*-[2-hydroxyethyl]-*N*-butylamino)diazen-1-ium-1,2,-diolate (**53d**). The oily residue (3.25 g) was comprised of iodomethyl acetate (**50**) (46%) and chloromethyl acetate (**49**) (54%) (calculated yield: 1.99 g [34%]). The proportion was calculated by comparing the ^{1}H NMR integration of the XCH_2O protons [^{1}H NMR ($CDCl_3$): chloromethyl acetate (**49**), δ 2.12 (s, 3H, Me), 5.74 (s, 2H, $ClCH_2O$); iodomethyl acetate (**50**), δ 2.12 (s, 3H, Me), 5.90 (s, 2H, ICH_2O)]. The spectral data matched those reported by Velázquez and Knaus (2004).

3.1.4.2 General Method for the Synthesis of O^2-Sodium 1-(*N*-[2-Hydroxyethyl]-*N*-alkylamino)diazen-1-ium-1,2,-diolates (52c-d)

O^2-Sodium 1-(*N*-[2-hydroxyethyl]-*N*-ethylamino)diazen-1-ium-1,2,-diolate (**52c**) and O^2-sodium 1-(*N*-[2-hydroxyethyl]-*N*-(*n*-butylamino)diazen-1-ium-1,2,-diolate (**52d**) were prepared from the corresponding *N*-(2-hydroxyethyl)-*N*-ethylamine (**51c**) and *N*-(2-hydroxyethyl)-*N*-butylamine (**51d**) by following the method described by Velázquez and Knaus (2004) for synthesizing O^2-sodium 1-(*N*-[2-hydroxyethyl]-*N*-methylamino)diazen-1-ium-1,2,-diolate and O^2-sodium 1-(4-[2-hydroxyethyl]piperazin-1-yl)diazen-1-ium-1,2,-diolate. Upon isolation, the unstable products were immediately used for the corresponding synthesis of O^2-acetoxymethyl 1-(*N*-[2-hydroxyethyl]-*N*-ethylamino)diazen-1-ium-1,2,-diolate (**53c**) and O^2-acetoxymethyl 1-(*N*-[2-hydroxyethyl]-*N*-(*n*-butylamino)diazen-1-ium-1,2,-diolate (**53d**). Yield (oil): **52c**, 82%; **52d**, 52%.

3.1.4.3 General Method for the Synthesis of O^2-Acetoxymethyl 1-(*N*-[2-Hydroxyethyl]-*N*-alkylamino)diazen-1-ium-1,2,-diolates (53c-d)

O^2-Acetoxymethyl 1-(*N*-[2-hydroxyethyl]-*N*-ethylamino)diazen-1-ium-1,2,-diolate (**53c**) and O^2-acetoxymethyl 1-(*N*-[2-hydroxyethyl]-*N*-[*n*-butylamino])diazen-1-ium-1,2,-diolate (**53d**) were prepared by modification of previously reported procedures [Velázquez & Knaus, 2004]. At 22°C in the dark and under an argon atmosphere,

sodium carbonate (1.47 g, 14 mmol) and then an O^2-sodium 1-(*N*-[2-hydroxyethyl]-*N*-alkylamino)diazen-1-ium-1,2,-diolate (**52c-d**) (12 mmol: **52c**, 2.04 g; **52d**, 2.37 g) were added to a solution of iodomethyl acetate (**50**) (1.99 g, 10 mmol) in acetone (40 mL). After 2 days of stirring, the mixture was exposed to air for 5 minutes and the solvent was removed *in vacuo*. Dichloromethane (30 mL) was added and the mixture was stored at 5°C for 1 hour. The insoluble inorganic salts were removed by filtering through filter paper, and the solvent was removed *in vacuo*. The oily residue was purified by silica gel column chromatography using 5% methanol in chloroform as eluant. The percent yield and spectral data for products **53c-d** are listed below.

3.1.4.3.1 O^2-Acetoxymethyl 1-(*N*-[2-Hydroxyethyl]-*N*-ethylamino)diazen-1-ium-1,2,-diolate (53c)

Yield: 30% (oil); ^{1}H NMR ($CDCl_3$): δ 1.14 (t, J = 7.0 Hz, 3H, NCH_2CH_3), 2.12 (s, 3H, acetoxy Me), 3.30 (q, J = 7.0 Hz, 2H, NCH_2CH_3), 3.34 (t, J = 4.9 Hz, 2H, OCH_2CH_2N), 3.69 (t, J = 4.9 Hz, 2H, OCH_2CH_2N), 5.81 (s, 2H, OCH_2O).

3.1.4.3.2 O^2-Acetoxymethyl 1-(*N*-[2-Hydroxyethyl]-*N*-[*n*-butyl]amino)diazen-1-ium-1,2,-diolate (53d)

Yield: 40% (oil); ^{1}H NMR ($CDCl_3$): δ 0.93 (t, J = 7.2 Hz, 3H, $NCH_2CH_2CH_2CH_3$), 1.38 (sextet, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 1.49 (quintet, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 2.12 (s, 3H, acetoxy Me), 3.23 (t, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 3.34 (t, J = 5.2 Hz, 2H, OCH_2CH_2N), 3.70 (t, J = 5.2 Hz, 2H, OCH_2CH_2N), 5.81 (s, 2H, OCH_2O).

3.1.4.4 General Method for the Synthesis of O^2-Acetoxymethyl 1-(*N*-[2-Methylsulfonyloxyethyl]-*N*-alkylamino)diazen-1-ium-1,2,-diolates (54c-d)

O^2-Acetoxymethyl 1-(*N*-[2-methylsulfonyloxyethyl]-*N*-ethylamino)diazen-1-ium-1,2,-diolate (**54c**) and O^2-acetoxymethyl 1-(*N*-[2-methylsulfonyloxyethyl]-*N*-butylamino)diazen-1-ium-1,2,-diolate (**54d**) were prepared by modification of previously reported procedures [Velázquez & Knaus, 2004]. At 22°C under an argon atmosphere, methanesulfonyl chloride (1.20 g, 10 mmol) and then DMAP (1.28 g, 10 mmol) were

slowly added to a solution of an O^2-acetoxymethyl 1-(*N*-[2-hydroxyethyl]-*N*-alkylamino)diazen-1-ium-1,2,-diolate (**53c-d**) (2.6 mmol: **53c**, 580 mg; **53d**, 654 mg) in acetonitrile (10 mL). After 20 hours of stirring, the solvent was removed *in vacuo*. Dichloromethane (50 mL) was added and the precipitates were removed by filtering through filter paper. After the solvent was removed *in vacuo*, the oily residue was purified by silica gel column chromatography using 10% (**54c**) and 20% (**54d**) ethyl acetate in chloroform as eluant. The unstable compounds were immediately used for the preparation of **60-65**. The percent yield and spectral data for products **54c-d** are listed below.

3.1.4.4.1 O^2-Acetoxymethyl 1-(*N*-[2-Methylsulfonyloxyethyl]-*N*-ethylamino)diazen-1-ium-1,2,-diolate (54c)

Yield: 56% (oil); ^{1}H NMR ($CDCl_3$): δ 1.16 (t, J = 7.2 Hz, 3H, NCH_2CH_3), 2.13 (s, 3H, acetoxy Me), 3.07 (s, 3H, $MeSO_2$), 3.30 (q, J = 7.2 Hz, 2H, NCH_2CH_3), 3.57 (t, J = 5.2 Hz, 2H, OCH_2CH_2N), 4.38 (t, J = 5.2 Hz, 2H, OCH_2CH_2N), 5.81 (s, 2H, OCH_2O).

3.1.4.4.2 O^2-Acetoxymethyl 1-(*N*-[2-Methylsulfonyloxyethyl]-*N*-[*n*-butyl]amino)diazen-1-ium-1,2,-diolate (54d)

Yield: 43% (oil); ^{1}H NMR ($CDCl_3$): δ 0.94 (t, J = 7.3 Hz, 3H, $NCH_2CH_2CH_2CH_3$), 1.38 (sextet, J = 7.3 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 1.51 (quintet, J = 7.3 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 2.12 (s, 3H, acetoxy Me), 3.07 (s, 3H, $MeSO_2$), 3.31 (t, J = 7.3 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 3.58 (t, J = 5.2 Hz, 2H, OCH_2CH_2N), 4.37 (t, J = 5.2 Hz, 2H, OCH_2CH_2N), 5.81 (s, 2H, OCH_2O).

3.1.4.5 General Method for the Synthesis of 3-Isopropyl 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (55a-c)

3-Isopropyl 5-(2-cyanoethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**55a-c**) were prepared according to the general method described in Section 3.1.3.4. A solid precipitated *in situ*. After removing the solvent *in vacuo*, the solid was washed with diethyl ether (3 × 30mL). The percent yield, melting point and spectral data for products **55a-c** are listed below.

3.1.4.5.1 3-Isopropyl 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (55a)

Yield: 28% (oil); mp: 146-147°C; ^{1}H NMR ($CDCl_3$): δ 1.10 and 1.23 (two d, J = 6.3 Hz, 3H each, isopropyl Me's), 2.26 and 2.27 (two s, 3H each, C-2 and C-6 Me's), 2.61 and 2.63 (two dd, J_{gem} = 9.9, J_{vic} = 5.3 Hz, 1H each, CH_aH$_b$N), 4.22 and 4.26 (two dd, J_{gem} = 9.9, J_{vic} = 5.3 Hz, 1H each, CH_aH$_b$O), 4.95 (septet, J = 6.3 Hz, 1H, isopropyl CH), 5.18 (s, 1H, H-4), 7.15 (ddd, $J_{4,5}$ = 8.1, $J_{5,6}$ = 4.8, $J_{3,5}$ = 0.5 Hz, 1H, pyridinyl H-5), 7.45 (d, $J_{3,4}$ = 8.3 Hz, 1H, pyridinyl H-3), 7.62 (ddd, $J_{3,4}$ = 8.3, $J_{4,5}$ = 8.1, $J_{4,6}$ = 0.9 Hz, 1H, pyridinyl H-4), 8.49 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6), 8.59 (sharp s, 1H, NH).

3.1.4.5.2 3-Isopropyl 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (55b)

Yield: 46% (oil); mp: 180-181°C (lit. mp: 192°C [Wehinger *et al.*, 1980]); ^{1}H NMR (DMSO-d_6): δ 1.03 and 1.18 (two d, J = 6.2 Hz, 3H each, isopropyl Me's), 2.28 and 2.29 (two s, 3H each, C-2 and C-6 Me's), 2.82 and 2.84 (two dd, J_{gem} = 11.5, J_{vic} = 5.8 Hz, 1H each, CH_aH$_b$N), 4.13 and 4.14 (two dd, J_{gem} = 11.5, J_{vic} = 5.8 Hz, 1H each, CH_aH$_b$O), 4.81 (septet, J = 6.2 Hz, 1H, isopropyl CH), 4.85 (s, 1H, H-4), 7.24 (dd, $J_{4,5}$ = 8.7, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-5), 7.53 (dd, $J_{4,5}$ = 8.7, $J_{4,6}$ = 2.6 Hz, 1H, pyridinyl H-4), 8.30 (d, $J_{4,6}$ = 2.6, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-6), 8.41 (d, $J_{2,4}$ = 1.9 Hz, 1H, pyridinyl H-2), 8.98 (sharp s, 1H, NH).

3.1.4.5.3 3-Isopropyl 5-(2-Cyanoethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (55c)

Yield: 39% (oil); mp: 151-152°C; ^{1}H NMR ($CDCl_3$): δ 1.13 and 1.26 (two d, J = 6.2 Hz, 3H each, isopropyl Me's), 2.36 and 2.38 (two s, 3H each, C-2 and C-6 Me's), 2.63 and 2.64 (two dd, J_{gem} = 10.5, J_{vic} = 6.1 Hz, 1H each, CH_aH$_b$N), 4.26 and 4.28 (two dd, J_{gem} = 12.3, J_{vic} = 6.1 Hz, 1H each, CH_aH$_b$O), 4.96 (septet, J = 6.2 Hz, 1H, isopropyl CH), 5.00 (s, 1H, H-4), 6.06 (sharp s, 1H, NH), 7.24 (d, $J_{2,3}$ = $J_{5,6}$ = 6.9 Hz, 2H, pyridinyl H-3 and H-5), 8.46 (d, $J_{2,3}$ = $J_{5,6}$ = 6.9 Hz, 2H, pyridinyl H-2 and H-6).

3.1.4.6 General Method for the Synthesis of 1,4-Dihydro-2,6-dimethyl-3-isopropyloxycarbonyl-4-(pyridinyl)-5-pyridinecarboxylic Acids (56a-c)

Twenty-five pecent (w/w) sodium methoxide in methanol (1.32 g, 24 mmol) chilled to 5°C was added drop-wise to a suspension of a 3-isopropyl 5-(2-cyanoethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylate (**55a-c**) (3.01 g, 8 mmol) in chloroform (10 mL) at 5°C. The mixture was stirred at 5°C for 5 minutes, and then 22°C for 3 d. Removal of the solvent *in vacuo* gave a residue that was dissolved in ice-water (10 mL) and then acidified with sulfuric acid (2 N) to pH 5.5. The precipitates were filtered and washed with diethyl ether. The percent yield, melting point and spectral data for products **56a-c** are listed below.

3.1.4.6.1 1,4-Dihydro-2,6-dimethyl-3-isopropyloxycarbonyl-4-(2-pyridinyl)-5-pyridinecarboxylic Acid (56a)

Yield: 98% (solid); mp: 185-186°C; ^{1}H NMR (DMSO-d_6): δ 0.93 and 1.12 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 2.18 and 2.19 (two s, 3H each, C-2 and C-6 Me's), 4.74 (septet, J = 6.1 Hz, 1H, isopropyl CH), 5.17 (s, 1H, H-4), 6.99 (ddd, $J_{4,5}$ = 8.1, $J_{5,6}$ = 4.8, $J_{3,5}$ = 0.5 Hz, 1H, pyridinyl H-5), 7.21 (d, $J_{3,4}$ = 8.3 Hz, 1H, pyridinyl H-3), 7.48 (ddd, $J_{3,4}$ = 8.3, $J_{4,5}$ = 8.1, $J_{4,6}$ = 0.9 Hz, 1H, pyridinyl H-4), 7.82 (sharp s, 1H, NH), 8.30 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6).

3.1.4.6.2 1,4-Dihydro-2,6-dimethyl-3-isopropyloxycarbonyl-4-(3-pyridinyl)-5-pyridinecarboxylic Acid (56b)

Yield: 88% (solid); mp: 191-193°C (lit. mp: 208°C; [Wehinger & Bossert, 1980]); ^{1}H NMR ($CDCl_3$): δ 0.99 and 1.15 (two d, J = 6.4 Hz, 3H each, isopropyl Me's), 2.26 and 2.27 (two s, 3H each, C-2 and C-6 Me's), 4.84 (septet, J = 6.2 Hz, 1H, isopropyl CH), 4.91 (s, 1H, H-4), 7.07 (dd, $J_{4,5}$ = 8.7, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-5), 7.22 (sharp s, 1H, NH), 7.57 (dd, $J_{4,5}$ = 8.7, $J_{4,6}$ = 2.6 Hz, 1H, pyridinyl H-4), 8.25 (d, $J_{4,6}$ = 2.6, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-6), 8.47 (d, $J_{2,4}$ = 1.9 Hz, 1H, pyridinyl H-2). *Anal.* Calcd. for $C_{17}H_{20}N_2O_4 \bullet 1/4H_2O$: C 63.63, H 6.29, N 8.73. Found: C 63.94, H 6.34, N 8.90. *m/z* (ES+) (Figure 3.1.4.6.2).

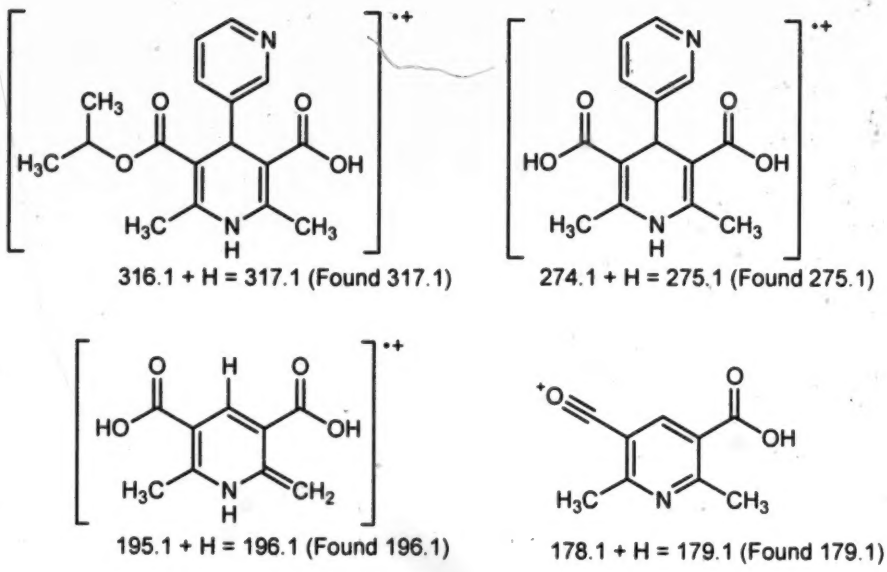


Figure 3.1.4.6.2: Fragmentation Pattern of 1,4-Dihydro-2,6-dimethyl-3-isopropyloxycarbonyl-4-(3-pyridinyl)-5-pyridinecarboxylic Acid (**56b**)

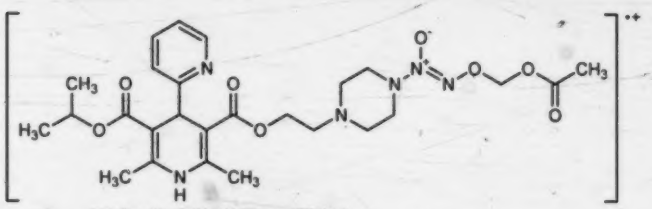
3.1.4.6.3 1,4-Dihydro-2,6-dimethyl-3-isopropyloxycarbonyl-4-(4-pyridinyl)-5-pyridinecarboxylic Acid (56c)

Yield: 50% (solid); mp: 198-199°C; ^{1}H NMR ($CDCl_3$): δ 1.03 and 1.17 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 2.27 and 2.28 (two s, 3H each, C-2 and C-6 Me's), 4.88 (septet, J = 6.1 Hz, 1H, isopropyl CH), 4.94 (s, 1H, H-4), 7.03 (broad s, 1H, NH), 7.18 (d, $J_{2,3} = J_{5,6}$ = 6.1 Hz, 2H, pyridinyl H-3 and H-5), 8.33 (d, $J_{2,3} = J_{5,6}$ = 6.1 Hz, 2H, pyridinyl H-2 and H-6).

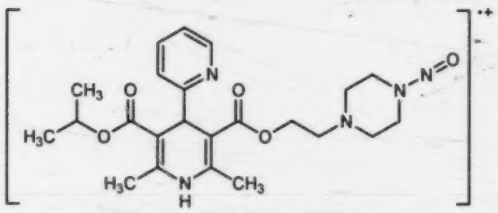
3.1.4.7 3-Isopropyl 5-(2-[4-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)piperazin-1-yl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (57)

Sodium carbonate (32 mg, 0.30 mmol) was added to a 1,4-dihydro-2,6-dimethyl-3-isopropyloxycarbonyl-4-(2-pyridinyl)-5-pyridinecarboxylic acid (**56a**) (79 mg, 0.25 mmol) in acetonitrile (5 mL) at 22°C, and the mixture was stirred for 30 minutes. O^2-acetoxymethyl 1-(4-[2-hydroxyethyl]piperazin-1-yl)diazen-1-ium-1,2,-diolate (**54a**) was added and the mixture was stirred at 70°C for 2 days. After removing the solvent *in*

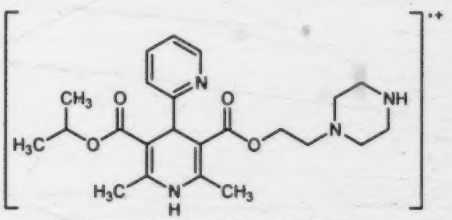
vacuo, ethyl acetate (70 mL) was added to the residue. The suspension was washed with water (3 × 30 mL), dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*. The residue was partially purified by PTLC using 5% methanol in chloroform as eluant. The residue was further purified by silica gel column chromatography using 5% methanol in chloroform as eluant.



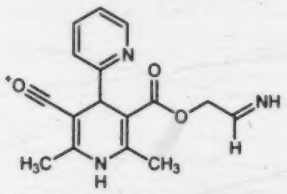
460.3 + Na = 583.3 (Found 583.3)
460.3 + H = 561.3 (Found 561.3)



457.2 + H = 458.2 (Found 458.3)



428.2 + H = 429.2 (Found 429.2)



298.1 + H = 299.1 (Found 299.2)

Figure 3.1.4.7: Fragmentation Pattern of 3-Isopropyl 5-(2-[4-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)piperazin-1-yl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**57**)

Yield: 16% (oil); IR ($CHCl_3$): 3436 (NH), 1703 (C=O), 1427 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.09 and 1.21 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 2.12 (s, 3H, acetoxy Me), 2.24 and 2.25 (two s, 3H each, C-2 and C-6 Me's), 2.58 – 2.63 (m, J_{gem} = unknown, $J_{vic(ethyl\ CH2N)}$ = 5.5, $J_{vic(piperazinyl\ H-2\ and\ H-6)}$ = 4.6 Hz, 3 × 2H, ethyl CH_2N and piperazinyl H-2 and H-6), 3.40 – 3.41 (m, J_{gem} = unknown, J_{vic} = 4.6 Hz, 2 × 2H, piperazinyl H-3 and H-5), 4.13 – 4.17 (m, J_{gem} = unknown, J_{vic} = 5.5 Hz, 2H, ethyl CH_2O), 4.94 (septet, J = 6.1 Hz, 1H, isopropyl CH), 5.16 (s, 1H, H-4), 5.79 (s, 2H, acetoxymethyl

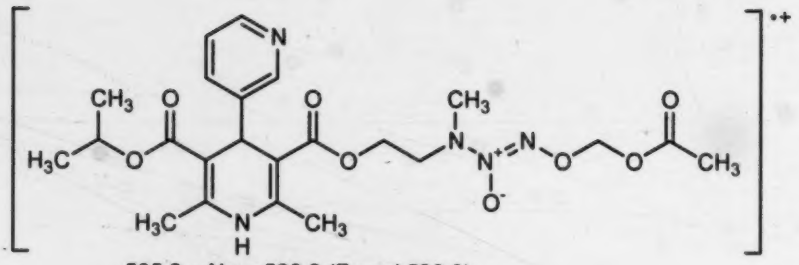
OCH_2O), 7.13 (ddd, $J_{4,5}$ = 8.1, $J_{5,6}$ = 4.8, $J_{3,5}$ = 0.5 Hz, 1H, pyridinyl H-5), 7.42 (d, $J_{3,4}$ = 8.3 Hz, 1H, pyridinyl H-3), 7.58 (ddd, $J_{3,4}$ = 8.3, $J_{4,5}$ = 8.1, $J_{4,6}$ = 0.9 Hz, 1H, pyridinyl H-4), 8.50 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6), 8.52 (sharp s, 1H, NH). *Anal.* Calcd. for $C_{26}H_{36}N_6O_8$ (not within ±0.4%): C 55.70, H 6.47, N 14.99. Found: C 42.31, H 4.78, N 9.78. *m/z* (ES+) (Figure 3.1.4.7).

3.1.4.8 General Method for the Synthesis of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-alkylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (58-65)

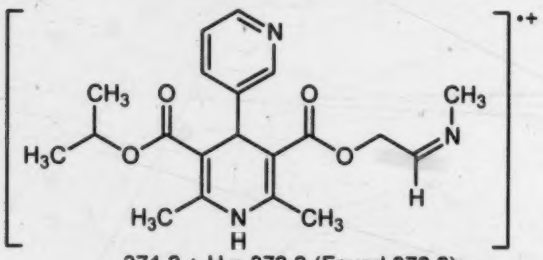
Sodium carbonate (42 mg, 0.40 mmol) was added to a 1,4-dihydro-2,6-dimethyl-3-isopropyloxycarbonyl-4-(pyridinyl)-5-pyridinecarboxylic acid (**56a-c**) (105 mg, 0.33 mmol) in acetonitrile (5 mL) at 22°C, and the mixture was stirred for 30 minutes. An O^2-acetoxymethyl 1-(*N*-[2-methylsulfonyloxyethyl]-*N*-alkylamino)diazen-1-ium-1,2,-diolate (**54b-d**) was added and the mixture was stirred at 50°C for 5 days. After removing the solvent *in vacuo*, dichloromethane (25 mL) was added to the residue. Insoluble solids were filtered by filter paper, washed with dichloromethane (2 × 25 mL) and discarded. The solvent was removed *in vacuo*, and the residue was purified by silica gel column chromatography using 3% methanol in chloroform as eluant. The percent yield and spectral data for products **58-65** are listed below.

3.1.4.8.1 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-methylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (58)

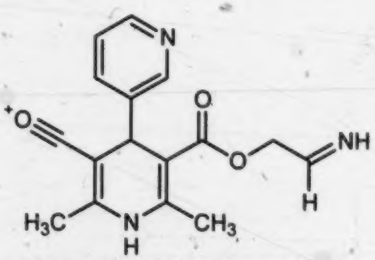
Yield: 40% (oil); IR ($CHCl_3$): 3429 (NH), 1696 (C=O), 1484 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.12 and 1.25 (two d, *J* = 6.4 Hz, 3H each, isopropyl Me's), 2.11 (s, 3H, acetoxy Me), 2.33 and 2.34 (two s, 3H each, C-2 and C-6 Me's), 3.00 (s, 3H, NMe), 3.62 (t, *J* = 5.5 Hz, 2H, OCH_2CH_2N), 4.23 (t, *J* = 5.5 Hz, 2H, OCH_2CH_2N), 4.94 (overlap of septet, *J* = 6.4 Hz, 1H, isopropyl CH; and s, 1H, H-4), 5.76 (s, 2H, OCH_2O), 6.34 (sharp s, 1H, NH), 7.17 (dd, $J_{4,5}$ = 8.7, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-5), 7.60 (dd, $J_{4,5}$ = 8.7, $J_{4,6}$ = 2.6 Hz, 1H, pyridinyl H-4), 8.36 (d, $J_{4,6}$ = 2.6, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-6), 8.51 (d, $J_{2,4}$ = 1.9 Hz, 1H, pyridinyl H-2). *Anal.* Calcd. for $C_{23}H_{31}N_5O_8$ (not within ±0.4%): C 54.65, H 6.18, N 13.85. Found: C 43.00, H 4.84, N 9.72. *m/z* (ES+) (Figure 3.1.4.8.1).



505.2 + Na = 528.2 (Found 528.3)
505.2 + H = 506.2 (Found 506.2)



371.2 + H = 372.2 (Found 372.2)

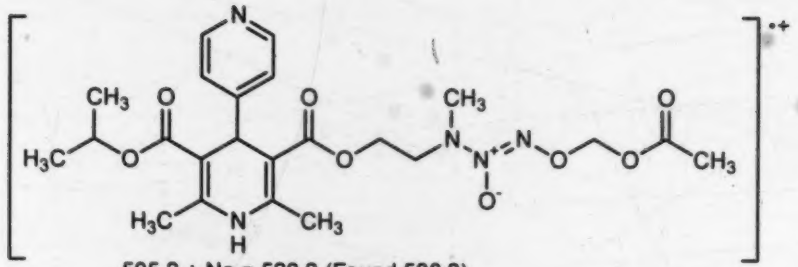


298.1 + H = 299.1 (Found 299.2)

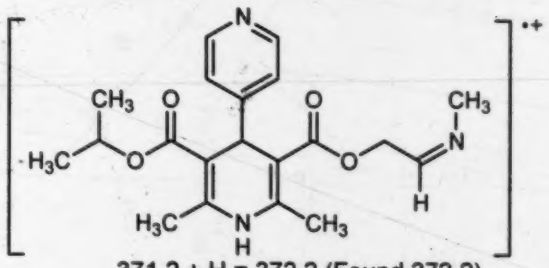
Figure 3.1.4.8.1: Fragmentation Pattern of 3-Isopropyl 5-(2-[*N*-(*O*^{2}-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-methylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (**58**)

3.1.4.8.2 3-Isopropyl 5-(2-[*N*-(*O*^{2}-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-methylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (59)

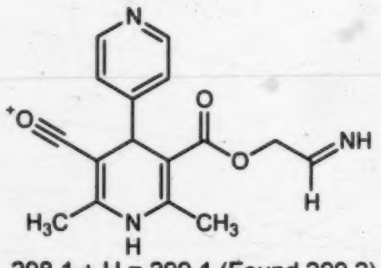
Yield: 33% (oil); IR ($CHCl_3$): 3436 (NH), 1683 (C=O), 1427 (NO_2) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.14 and 1.26 (two d, J = 6.3 Hz, 3H each, isopropyl Me's), 2.12 (s, 3H, acetoxy Me), 2.34 and 2.36 (two s, 3H each, C-2 and C-6 Me's), 3.00 (s, 3H, NMe), 3.62 (t, J = 5.5 Hz, 2H, OCH_2CH_2N), 4.25 (t, J = 5.5 Hz, 2H, OCH_2CH_2N), 4.98 (overlap of septet, J = 6.3 Hz, 1H, isopropyl CH; and s, 1H, H-4), 5.76 (s, 2H, OCH_2O), 6.0 (sharp s, 1H, NH), 7.23 (d, $J_{2,3} = J_{5,6}$ = 5.6 Hz, 2H, pyridinyl H-3 and H-5), 8.43 (d, $J_{2,3} = J_{5,6}$ = 5.6 Hz, 2H, pyridinyl H-2 and H-6). *Anal.* Calcd. for $C_{23}H_{31}N_5O_8$ (not within ±0.4%): C 54.65, H 6.18, N 13.85. Found: C 43.23, H 4.84, N 9.74. *m/z* (ES+) (Figure 3.1.4.8.2).



505.2 + Na = 528.2 (Found 528.2)
505.2 + H = 506.2 (Found 506.2)



371.2 + H = 372.2 (Found 372.2)

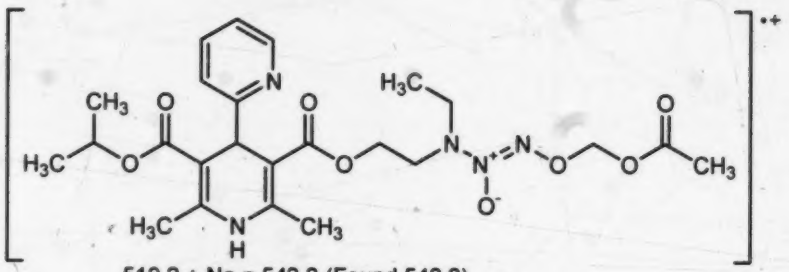


298.1 + H = 299.1 (Found 299.2)

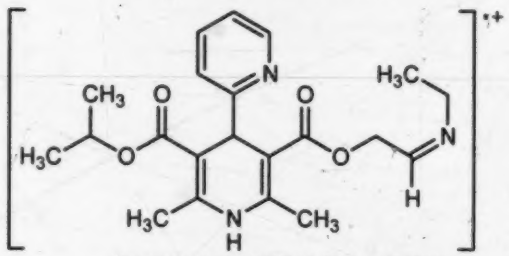
Figure 3.1.4.8.2: Fragmentation Pattern of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-methylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (**59**)

3.1.4.8.3 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (60)

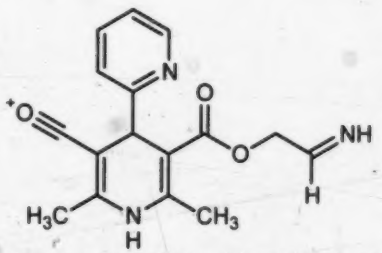
Yield: 52% (oil); IR ($CHCl_3$): 3449 (NH), 1703 (C=O), 1501 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.07 and 1.20 (two d, J = 6.2 Hz, 3H each, isopropyl Me's), 1.07 (t, J = 7.0 Hz, NCH_2CH_3), 2.08 (s, 3H, acetoxy Me), 2.21 (s, 2 × 3H, C-2 and C-6 Me's), 3.44 (q, 2H, J = 7.0 Hz, 2H, NCH_2CH_3), 3.44 (t, J = 5.5 Hz, 2H, OCH_2CH_2N), 4.16 (t, J = 5.5 Hz, 2H, OCH_2CH_2N), 4.92 (septet, J = 6.2 Hz, 1H, isopropyl CH), 5.15 (s, 1H, H-4), 5.76 (s, 2H, OCH_2O), 7.14 (ddd, $J_{4,5}$ = 8.1, $J_{5,6}$ = 4.8, $J_{3,5}$ = 0.5 Hz, 1H, pyridinyl H-5), 7.42 (d, $J_{3,4}$ = 8.3 Hz, 1H, pyridinyl H-3), 7.59 (ddd, $J_{3,4}$ = 8.3, $J_{4,5}$ = 8.1, $J_{4,6}$ = 0.9 Hz, 1H, pyridinyl H-4), 8.46 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6), 9.12 (sharp s, 1H, NH). *Anal.* Calcd. for $C_{24}H_{33}N_5O_8$ (not within ±0.4%): C 55.48, H 6.40, N 13.48. Found: C 49.71, H 5.48, N 11.08. *m/z* (ES+) (Figure 3.1.4.8.3).



519.2 + Na = 542.2 (Found 542.2)
519.2 + H = 520.2 (Found 520.3)



385.2 + H = 386.2 (Found 386.2)



298.1 + H = 299.1 (Found 299.1)

Figure 3.1.4.8.3: Fragmentation Pattern of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**60**)

3.1.4.8.4 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (61)

Yield: quantitative (oil); IR ($CHCl_3$): 3435 (NH), 1691 (C=O), 1494 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.09 and 1.24 (two d, J = 6.3 Hz, 3H each, isopropyl Me's), 1.10 (t, J = 7.0 Hz, NCH_2CH_3), 2.09 (s, 3H, acetoxy Me), 2.32 (s, 2 × 3H, C-2 and C-6 Me's), 3.27 (q, 2H, J = 7.0 Hz, 2H, NCH_2CH_3), 3.44 (t, J = 5.6 Hz, 2H, OCH_2CH_2N), 4.18 (t, J = 5.6 Hz, 2H, OCH_2CH_2N), 4.94 (overlap of septet, J = 6.3 Hz, 1H, isopropyl CH; and s, 1H, H-4), 5.76 (s, 2H, OCH_2O), 6.68 (broad s, 1H, NH), 7.16 (dd, $J_{4,5}$ = 8.7, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-5), 7.63 (dd, $J_{4,5}$ = 8.7, $J_{4,6}$ = 2.6 Hz, 1H, pyridinyl H-4), 8.35 (d, $J_{4,6}$ = 2.6, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-6), 8.51 (d, $J_{2,4}$ = 1.9 Hz, 1H, pyridinyl H-2). *m/z* (ES+) (Figure 3.1.4.8.4).

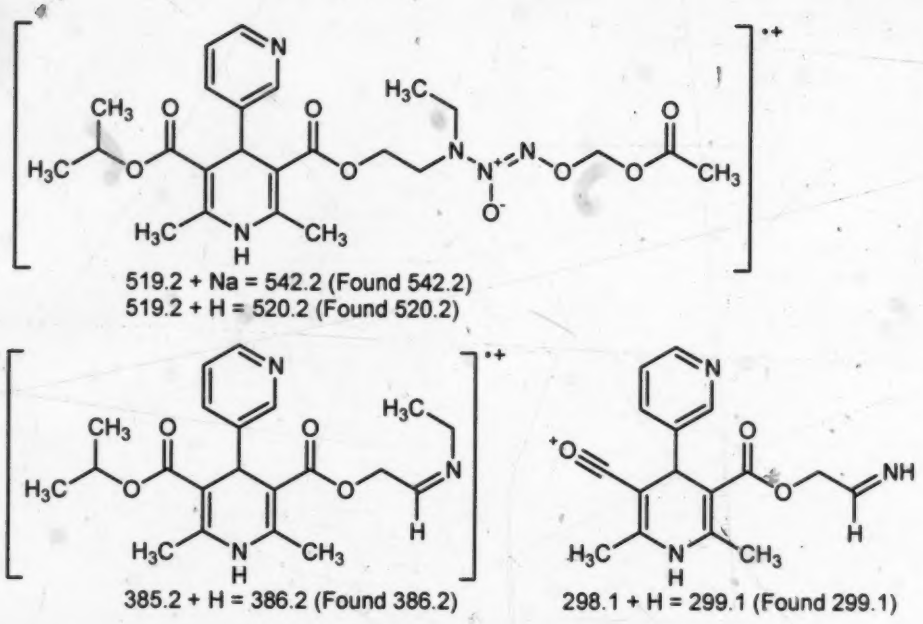


Figure 3.1.4.8.4: Fragmentation Pattern of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (**61**)

3.1.4.8.5 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (62)

Yield: quantitative (oil); IR ($CHCl_3$): 3436 (NH), 1696 (C=O), 1494 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.06 (t, J = 7.0 Hz, NCH_2CH_3), 1.10 and 1.23 (two d, J = 6.3 Hz, 3H each, isopropyl Me's), 2.09 (s, 3H, acetoxy Me), 2.32 (s, 2 × 3H, C-2 and C-6 Me's), 3.26 (q, 2H, J = 7.0 Hz, 2H, NCH_2CH_3), 3.45 (t, J = 5.5 Hz, 2H, OCH_2CH_2N), 4.19 (t, J = 5.5 Hz, 2H, OCH_2CH_2N), 4.96 (septet, J = 6.3 Hz, 1H, isopropyl CH), 4.97 (s, 1H, H-4), 5.75 (s, 2H, OCH_2O), 6.67 (broad s, 1H, NH), 7.23 (d, $J_{2,3} = J_{5,6}$ = 5.5 Hz, 2H, pyridinyl H-3 and H-5), 8.41 (d, $J_{2,3} = J_{5,6}$ = 5.5 Hz, 2H, pyridinyl H-2 and H-6). *m/z* (ES+) (Figure 3.1.4.8.5).

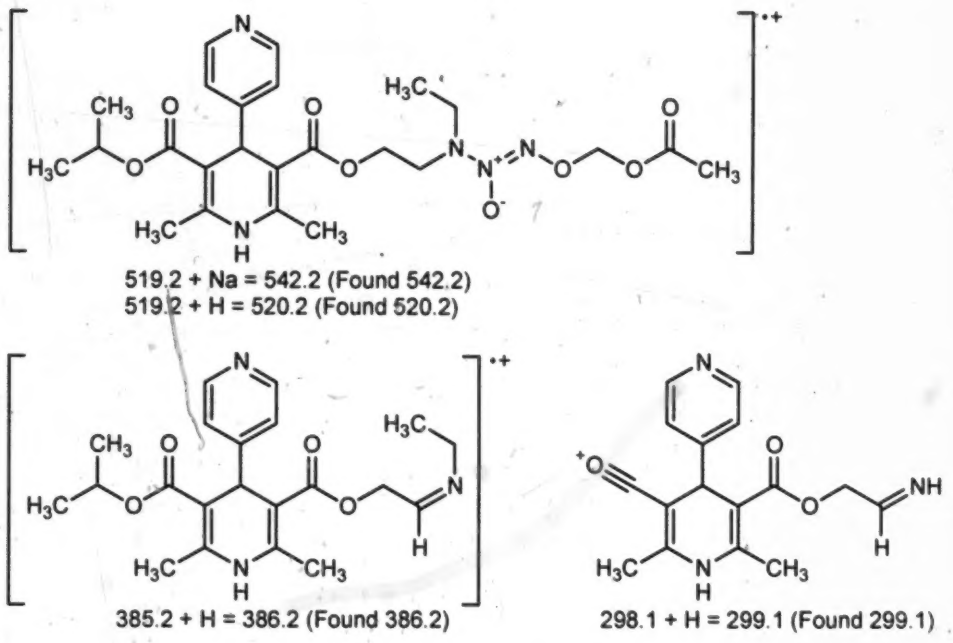


Figure 3.1.4.8.5: Fragmentation Pattern of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (**62**)

3.1.4.8.6 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-(*n*-butyl)amino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (63)

Yield: 71% (oil); IR ($CHCl_3$): 3436 (NH), 1690 (C=O), 1497 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.07 (t, J = 7.2 Hz, $NCH_2CH_2CH_2CH_3$), 1.08 and 1.21 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 1.31 (sextet, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 1.42 (quintet, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 2.09 (s, 3H, acetoxy Me), 2.23 (s, 2 × 3H, C-2 and C-6 Me's), 3.20 (t, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 3.45 (t, J = 5.3 Hz, 2H, OCH_2CH_2N), 4.16 (t, J = 5.3 Hz, 2H, OCH_2CH_2N), 4.93 (septet, J = 6.1 Hz, 1H, isopropyl CH), 5.15 (s, 1H, H-4), 5.77 (s, 1H, OCH_2O), 7.14 (ddd, $J_{4,5}$ = 8.1, $J_{5,6}$ = 4.8, $J_{3,5}$ = 0.5 Hz, 1H, pyridinyl H-5), 7.44 (d, $J_{3,4}$ = 8.3 Hz, 1H, pyridinyl H-3), 7.59 (ddd, $J_{3,4}$ = 8.3, $J_{4,5}$ = 8.1, $J_{4,6}$ = 0.9 Hz, 1H, pyridinyl H-4), 8.48 (d, $J_{5,6}$ = 4.8 Hz, 1H, pyridinyl H-6), 8.75 (broad s, 1H, NH).

Anal. Calcd. for $C_{26}H_{37}N_5O_8$ (not within ±0.4%): C 57.03, H 6.81, N 12.79. Found: C 51.67, H 6.36, N 10.11. *m/z* (ES+) (Figure 3.1.4.8.6).

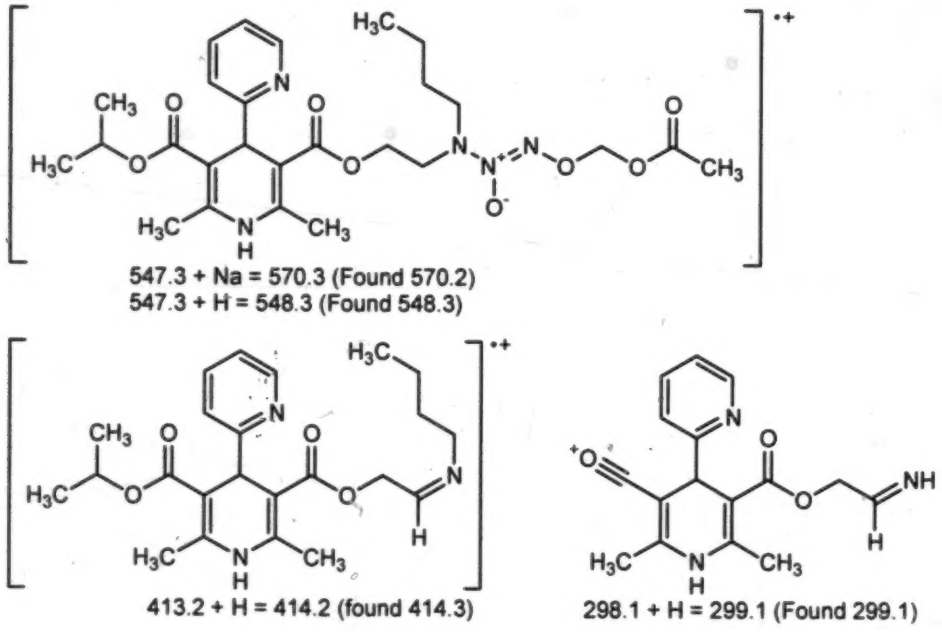


Figure 3.1.4.8.6: Fragmentation Pattern of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-(*n*-butyl)amino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**63**)

3.1.4.8.7 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-(*n*-butyl)amino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (64)

Yield: 46% (oil); IR ($CHCl_3$): 3432 (NH), 1690 (C=O), 1492 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.90 (t, *J* = 7.0 Hz, $NCH_2CH_2CH_2CH_3$), 1.10 and 1.26 (two d, *J* = 6.1 Hz, 3H each, isopropyl Me's), 1.31 (sextet, *J* = 7.0 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 1.42 (quintet, *J* = 7.0 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 2.10 (s, 3H, acetoxy Me), 2.33 (s, 2 × 3H, C-2 and C-6 Me's), 3.18 (t, *J* = 7.0 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 3.45 (t, *J* = 5.5 Hz, 2H, OCH_2CH_2N), 4.18 (t, *J* = 5.5 Hz, 2H, OCH_2CH_2N), 4.95 (overlap of septet, *J* = 6.1 Hz, 1H, isopropyl CH; and s, 1H, H-4), 5.77 (s, 1H, OCH_2O), 6.22 (broad s, 1H, NH), 7.18 (dd, $J_{4,5}$ = 8.7, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-5), 7.62 (dd, $J_{4,5}$ = 8.7, $J_{4,6}$ = 2.6 Hz, 1H, pyridinyl H-4), 8.40

(d, $J_{4,6}$ = 2.6, $J_{5,6}$ = 5.0 Hz, 1H, pyridinyl H-6), 8.52 (d, $J_{2,4}$ = 1.9 Hz, 1H, pyridinyl H-2). *m/z* (ES+) (Figure 3.1.4.8.7).

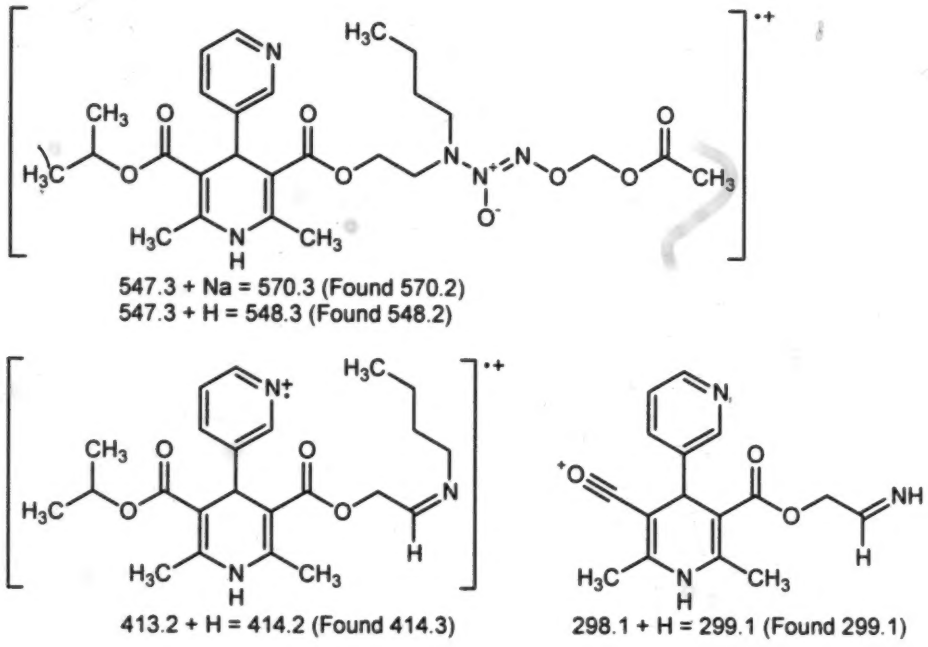
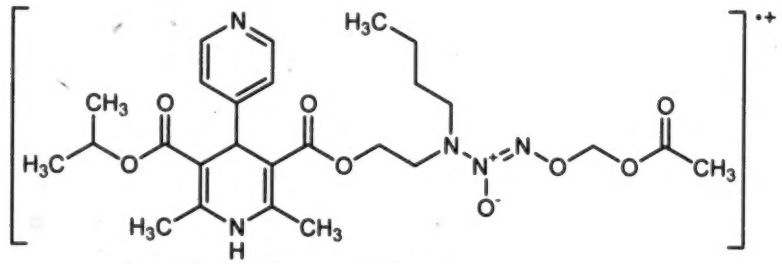


Figure 3.1.4.8.7: Fragmentation Pattern of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-(*n*-butyl)amino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (**64**)

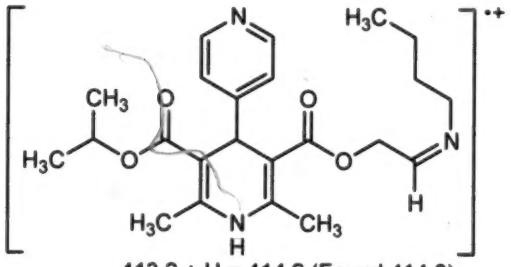
3.1.4.8.8 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-(*n*-butyl)amino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (65)

Yield: 47% (oil); IR ($CHCl_3$): 3442 (NH), 1691 (C=O), 1494 (NO_2) cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.90 (t, J = 7.2 Hz, $NCH_2CH_2CH_2CH_3$), 1.12 and 1.25 (two d, J = 6.1 Hz, 3H each, isopropyl Me's), 1.32 (sextet, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 1.43 (quintet, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 2.10 (s, 3H, acetoxy Me), 2.35 (s, 2 × 3H, C-2 and C-6 Me's), 3.18 (t, J = 7.2 Hz, 2H, $NCH_2CH_2CH_2CH_3$), 3.45 (t, J = 5.5 Hz, 2H, OCH_2CH_2N), 4.19 (t, J = 5.5 Hz, 2H, OCH_2CH_2N), 4.97 (overlap of septet, J = 6.1 Hz, 1H, isopropyl CH; and s, 1H, H-4), 5.76 (s, 1H, OCH_2O), 5.95 (broad s, 1H, NH), 7.23 (d, $J_{2,3}$ = $J_{5,6}$ =

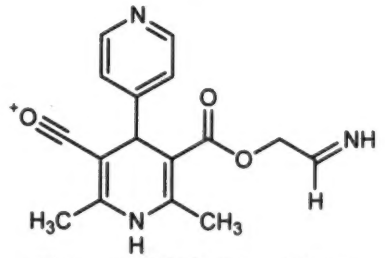
5.5 Hz, 2H, pyridinyl H-3 and H-5), 8.41 (d, $J_{2,3} = J_{5,6}$ = 5.5 Hz, 2H, pyridinyl H-2 and H-6). *m/z* (ES+) (Figure 3.1.4.8.8).



547.3 + Na = 570.3 (Found 570.2)
547.3 + H = 548.3 (Found 548.2)



413.2 + H = 414.2 (Found 414.3)



298.1 + H = 299.1 (Found 299.1)

Figure 3.1.4.8.8: Fragmentation Pattern of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-(*n*-butyl)amino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (**65**)

3.2 PHARMACOLOGY

3.2.1 *In Vitro* Calcium Channel Agonist and Antagonist Assays

Carol-Anne Schoettle and Carlos Velázquez performed the *in vitro* calcium channel agonist and antagonist assays according to previously reported procedures [Vo *et al.*, 1995]. The PhD candidate, Jeffrey-Tri Nguyen, was an observer. *In vitro* calcium channel antagonist and agonist activities were determined using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

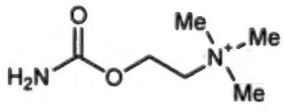


Figure 3.2.1: Carbachol

Smooth muscle calcium channel antagonist activity (IC_{50}) was defined as the molar concentration of the test compound required to produce 50% inhibition of the muscarinic receptor-mediated (carbachol, 0.167 μM) (Figure 3.2.1) calcium-dependent contraction (tonic response) of guinea pig ileum longitudinal smooth muscle. A tissue sample's IC_{50} value was determined graphically, when possible, from the dose-response curve. The average of all samples' IC_{50} values represented the test compound's smooth muscle antagonistic IC_{50} value (± SEM, n = 2 or 3).

Calcium channel agonist activity on guinea pig ileum longitudinal smooth muscle (in the absence of 0.167 μM carbachol) (EC_{50}) was defined as the molar concentration of the test compound required to elicit a 50% increase in the contractile response, relative to the response produced by carbachol (0.167 μM). A tissue sample's EC_{50} value was determined graphically, when possible, from the dose-response curve. The average of three samples' EC_{50} values represented the test compound's smooth muscle agonistic EC_{50} value (± SEM, n = 3).

The cardiac calcium channel agonist effect (EC_{50}) was calculated as the percentage increase (positive inotropic effect) in contractile force of an isolated guinea pig left atrium relative to its basal contractile force in the absence of the test compound. A tissue sample's EC_{50} value was determined graphically, when possible, from the dose-response curve. The average of three samples' EC_{50} values represented the test compound's cardiac positive inotropic EC_{50} value (± SEM, n = 3).

The cardiac chronotropic effect was calculated as the percentage increase in beats per minute of an isolated guinea pig right atrium relative to its basal heart rate in the absence of the test compound.

3.2.2 Nitric Oxide Release Assays

3.2.2.1 *In Vitro* Nitric Oxide Release Assay Using *N*-Acetylcysteamine

In vitro nitric oxide release in the presence of *N*-acetylcysteamine, determined by quantification of nitrite produced by the reaction of nitric oxide with oxygen and water, using the Griess reaction [Sako *et al.*, 1998; Cook *et al.*, 1996], was measured for the test compounds **6-17** and the reference drug, glyceryl trinitrate, using the following procedure.

Phosphate buffer solution was prepared by titrating $Na_2HPO_4 \cdot 7H_20$ (0.1 N) with H_3PO_4 (0.1 N) to physiological pH (7.40). The Griess reagent was prepared by

dissolving sulfanilamide (4.0 g) and *N*-(1-naphthyl)ethylenediamine•2HCl (0.2 g) in a mixture of H_3PO_4 (85% grade, 10 mL) and distilled water (90 mL).

A solution of $NaNO_2$ (60 μM) in acetonitrile and phosphate buffer (1:1 v/v) was maintained at 37°C under argon for 1 hour, then at 25°C in an air atmosphere for 10 minutes. Griess reagent (1.5 mL) was mixed with an aliquot of the above solution (1.5 mL) that was maintained at 25°C for 10 minutes. A standard nitrite-concentration curve, which correlates nitrite-concentration and absorbance, was prepared by dissolving the mixture prepared above with varying amounts of distilled water to provide the desired nitrite concentrations, and measuring the respective ultraviolet absorbance at 540 nm.

A solution of the test compound (2.5 mM, 3 mL) in acetonitrile and phosphate buffer (1:1 v/v) was prepared. An aliquot of this solution (1.5 mL) was used to determine any possible test compound absorption at 540 nm (baseline). A solution of *N*-acetylcysteamine in acetonitrile (20-60 μL, 1 equivalent per nitrooxy group in the test compound) was mixed with the second aliquot (1.5 mL) to determine nitrite concentration. The solution was maintained at 37°C under argon for 1 hour, at 25°C in the presence of air for 10 minutes, Griess reagent (1.5 mL) was mixed with each 1.5 mL test compound sample, this mixture was maintained at 25°C for 10 minutes, and then ultraviolet absorbance at 540 nm was measured. The absorbance was corrected by subtracting the test compound baseline absorbance, and the nitrite concentration was determined from the standard nitrite concentration curve to calculate the percent nitric oxide released from the test compound (mol / mol).

3.2.2.2 *In Vitro* Nitric Oxide Release Assay Using L-Cysteine

In vitro nitric oxide release in the presence of L-cysteine, determined by quantification of nitrite produced by the reaction of nitric oxide with oxygen and water using the Griess reaction [Fruttero *et al.*, 1995], was measured for the test compounds **24-38**, **46-48**, **57-65** and the reference drug, glyceryl trinitrate, using the following procedure.

Phosphate buffer containing L-cysteine (PBC) solution was prepared by mixing a solution of KH_2PO_4 (50 mN, 300 mL) and $NaHPO_4•7H_2O$ (50 mN, 300 mL). L-cysteine (364 mg, 3 mmol) was added and this solution was titrated to physiological pH (7.4) using KH_2PO_4 (50 mN, 285 mL). The final concentration of L-cysteine in this PBC solution was 3.4 mM. The Griess reagent was prepared by dissolving sulfanilamide (4.0 g) and *N*-(1-naphthyl)ethylenediamine•2HCl (0.2 g) in a mixture of H_3PO_4 (85% grade,

10 mL) and distilled water (90 mL). A solution of 5% dimethyl sulfoxide in PBC (5% DPBC solution) was prepared by diluting dimethyl sulfoxide (10 mL) with PBC solution (190 mL). An aliquot of this 5% DPBC solution (2.4 mL) was used as a control blank that was maintained at 37°C for 1 hour with gentle shaking. Griess reagent (0.8 mL) was added, the mixture was maintained at 37°C for 30 minutes with gentle shaking, and the solution's ultraviolet absorbance at 540 nm was measured (± SEM, n = 3).

A standard nitrite-absorbance concentration plot was prepared as followed. A solution of $NaNO_2$ (0.1 mM) in 5% DPBC solution (2.4 mL) was prepared, and this solution was maintained at 37°C for 1 hour with gentle shaking. Griess reagent (0.8 mL) was added and the mixture was maintained at 37°C for 30 minutes with gentle shaking. A solution of $NaNO_2$ for use as a dilution solvent (SNDS) was prepared by mixing Griess reagent (32 mL) with 5% DPBC solution (96 mL). Dilutions of the $NaNO_2$ containing solution with SNDS solution were used to prepare the calibration curve from which nitrite concentrations (absorbance at 540 nm) were calculated (± SEM, n = 3).

The percentage nitric oxide released from the test compounds was determined as follows. A solution of the test compound (2 mM) in dimethyl sulfoxide (5 mL) was prepared. An aliquot (0.12 mL) was diluted with PBC solution (2.28 mL) to provide a final concentration (0.1 mM) of the test compound in phosphate buffer solution containing 5% dimethyl sulfoxide and 3.2 mM L-cysteine (2.4 mL). This solution was maintained at 37°C for 1 hour with gentle shaking, Griess reagent (0.8 mL) was added, and the mixture was maintained at 37°C with gentle shaking for 30 minutes. The solution ultraviolet absorbance was measured at 540 nm. The absorbance was corrected by subtracting averaged blank absorbance. The nitrite concentration was determined from the standard nitrite concentration-absorbance curve to calculate the percent nitric oxide released from the test compound (mol / mol).

Nitric oxide release studies, in the absence of L-cysteine, were performed in a similar manner except that the phosphate buffer solution did not contain L-cysteine.

3.2.2.3 Nitric Oxide Release Assay in Guinea Pig Serum

Nitric oxide release in guinea pig serum, determined by quantification of nitrite produced by the reaction of nitric oxide with oxygen and water using the Griess reaction, was measured for the test compounds **57-65** and the reference drug, glyceryl trinitrate, using the following procedure.

After sacrificing guinea pigs, whole blood was immediately collected from their hearts. The blood was left standing at 5°C for 20 hours so as to permit the serum to separate. The blood was centrifuged at 2000 rpm for 15 minutes and the clear serum was moved into a clean test tube. The Griess reagent was prepared by dissolving sulfanilamide (4.0 g) and *N*-(1-naphthyl)ethylenediamine•2HCl (0.2 g) in a mixture of H_3PO_4 (85% grade, 10 mL) and distilled water (90 mL).

A standard nitrite-absorbance concentration plot was prepared as follows. A solution of $NaNO_2$ (25 mM) in acetonitrile and serum (5:95 v/v, 400 µL) was prepared by serial dilution ($NaNO_2$ [3.5 mg, 0.05 mmol] was dissolved in serum [200 µL]. A portion of this solution [40 µL] was diluted with serum [320 µL] and acetonitrile [20 µL] was mixed in). Eleven dilutions with acetonitrile and serum (5:95 v/v) were prepared from this solution (25 mM). An undiluted nitrite sample (25 mM), the eleven dilutions, and a nitrite-free serum sample (0 mM) (100 µL each) were incubated in a multiple-wells cuvette at 37°C with gentle shaking for 1 hour. Griess reagent (33 µL) was added to each sample, and the mixtures were incubated at 37°C with gentle shaking for 30 minutes. The solution ultraviolet absorbances were measured at 540 nm. The absorbances were used to prepare the calibration curve from which nitrite concentrations were calculated (± SEM, n = 3).

The percentage nitric oxide released from the test compounds was determined as followed. A solution of the test compound dissolved first in acetonitrile (20 µL) then serum (380 µL) (10 mM, 400 µL) was prepared. The solution was divided into 3 parts (100 µL each) and incubated in a multiple-wells cuvette at 37°C with gentle shaking for 1 hour. Griess reagent (33 µL) was added to each part, and the mixtures were incubated at 37°C with gentle shaking for 30 minutes. The solution ultraviolet absorbance was measured at 540 nm. The nitrite concentration was determined from the standard nitrite concentration-absorbance curve to calculate the percent nitric oxide released from the test compound (mol / mol).

3.3 *IN SILICO* METHODS

We developed two similar methods for deriving multiple collinear regression equations for our quantitative-structure activity relationship studies. Each method will be discussed in the Discussion Sections 4.3.1.2 and 4.3.2.3, because it is easier to describe each method from numerical examples.

Computer modelling and dynamic docking experiments were performed according to literature procedures [Zhorov *et al.*, 2001; Zamponi *et al.*, 2003].

4 RESULTS AND DISCUSSION

4.1 PREAMBLE

Among the three drug classes used to treat heart failure, namely diuretics, positive inotropic agents and vasodilators [Johnson *et al.*, 1999; Ooi & Colucci, 2001], studies were initiated to design groups of hybrid drugs that offer both benefits of a positive inotropic agent and a vasodilator. The 1,4-dihydropyridine groups of calcium channel modulators were of interest to us, because they can act either as an agonist and/or an antagonist. Indeed, we were interested in the properties of 1,4-dihydropyridine calcium channel agonists as positive inotropic agents and 1,4-dihydropyridine calcium channel antagonists as vasodilators.

Calcium channel modulators that act directly on the target tissues have fewer systemic side-effects [Triggle, 1992; Natale *et al.*, 1999; Kevins & Robertson, 2001]. Moreover, unlike other classes of calcium channel modulators such as the phenylalkylamine calcium channel antagonists from which verapamil is a representative example, or the benzothiazepine calcium channel antagonists such as diltiazem, 1,4-dihydropyridine calcium channel modulators actions are more specific for the L-type channels. Hence, when compared to the other classes of calcium channel modulators, 1,4-dihydropyridines calcium channel modulators do not detrimentally affect other physiological systems outside of the cardiovascular system.

In order to enhance the smooth muscle relaxant action of calcium channel antagonists, and abolish the smooth muscle contraction action of racemic calcium channel agonists[16], a nitric oxide donor group was covalently attached to a 1,4-dihydropyridine moiety. Accordingly, 1,4-dihydropyridines induce vascular smooth muscle relaxation, particularly in arterial beds, which is the basis for their therapeutic use in the treatment of peripheral vascular disorders, angina pectoris and hypertension [Fleckenstein, 1977]. In contrast, organic nitrates such as glyceryl trinitrate induce dilation of venous vessels that is primarily due to nitric oxide release [Mackenzie & Parratt, 1977]. Indeed, in animal studies done by other research groups, a combination of the calcium channel antagonist, nifedipine, and the nitric oxide donor, glyceryl trinitrate, showed a good pharmacological effect in oxygen saturation and oxygen pressure in the coronary sinus [Bossert & Vater, 1989]. In our studies, we want to

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[16] The results are discussed in Sections 4.3.1.3 and 4.3.2.2.2.

combine the beneficial properties of both drug classes into one molecule to dertermine whether the synergistic effect is restricted to organic nitrates[17].

Christiaans and Timmerman's 1996 review article revealed that there is considerable interest in cardiovascular hybrid drugs, as an alternative to combination drug therapy, which combines more than one pharmacological property in a single drug. A 1,4-dihydropyridine hybrid drug that has multiple independent mechanisms of action will greatly enhance its pharmacological effects or reduce its undesired side-effects.

One of the major drawbacks of calcium channel agonists is the lack of cardioselectivity [Holland *et al.*, 1989; Gross *et al.*, 1990]. Because the extent of cardioselectivity is difficult to predict, we are more interested in attaching a moiety that can counteract the vasocontractility of calcium channel agonists while preserving the positive inotropic effect, than finding means to enhance cardioselectivity. The addition of a nitric oxide donor moiety to the parent compound can counter-balance the vascular constriction effect induced by calcium channel agonists. Consequently, we wanted to design 1,4-dihydropyridine hybrids that can simultaneously release nitric oxide.

1,4-Dihydropyridine calcium channel antagonists can play an essential role in treating heart failure if non-cardioselective negative inotropic effect, sympathetic nervous system activation and reflex tachycardia properties can be removed from their pharmacological profiles [Johnson *et al.*, 1999; Kevins & Robertson, 2001; Ooi & Colucci, 2001]. It has been shown that nitrendipine facilitates the release of nitric oxide from vascular endothelium which may contribute to its vascular relaxation effect [Gunther *et al.*, 1992], that nitric oxide modulates the activity of the calcium ion release channel by preventing oxidation of regulatory sulfhydryls [Aghdasi *et al.*, 1997], that the nitric oxide/cyclic GMP pathway facilitates the inhibitory effect of calcium channel antagonists [Salomone *et al.*, 1996], and that nitric oxide donors evoke a small but constant positive inotropic effect *in vivo* that is not caused by coronary vasodilation [Preckel *et al.*, 1997]. Therefore, the addition of a nitric oxide donor moiety to the parent compound can enhance the vasodilatory effect of calcium channel antagonists, while alleviating the undesired pharmacological effects of calcium channel agonists.

Unlike 1,4-dihydropyridine calcium channel modulators that act directly at the site of action, nitrovasodilators release nitric oxide that mediates the contractility of vascular tissues [Kevins & Robertson, 2001; Ooi & Colucci, 2001]. Thus, although

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[17] The results are discussed in Sections 4.3.2.1 and 4.3.2.3.

nitrovasodilators may enhance the beneficial effects and reduce the detrimental effects of 1,4-dihydropyridines, nitric oxide, as a signaling agent, has an increased risk of inducing systemic side-effects such as migraine headaches or even hallucinations [Johnson *et al.*, 1999; Ooi & Colucci, 2001].

Consequently, we are interested in nitrovasodilators that gradually release nitric oxide or those that release nitric oxide at the site of action. Because a given amount of nitric oxide released slowly over a long period of time has a less potent but more prolonged duration of vasodilatory action then the same amount of nitric oxide released at once [Keefer, 1998], a sustained release nitrovasodilator is more beneficial as a homeostatic agent than an immediate release nitrovasodilator. Moreover, a gradual release of nitric oxide has a reduced risk of inducing migraine headaches and hallucinations. Additionally, by attaching a 1,4-dihydropyridine calcium channel modulation moiety to a nitrovasodilator, the released nitric oxide may be restricted to the 1,4-dihydropyridine calcium channel modulator's site of action, and may thereby be less likely to affect the central nervous system.

4.1.1 1,4-DIHYDROPYRIDINE CALCIUM CHANNEL MODULATORS

1,4-Dihydropyridine calcium channel modulators, when the C-3 and C-5 ester substituents are different, are chiral molecules in which case the chiral centre is located at the C-4 position. One enantiomer usually acts as a calcium channel agonist while the other acts as a calcium channel antagonist [Mahmoudian & Richards, 1986a; Triggle & Rampe, 1989; Triggle, 1992]. Because both the agonist and antagonist effects play potentially beneficial roles in treating heart failure, the difficult and tedious isolation of the enantiomers from the racemate is often not necessary in these preliminary studies.

To study the effect of different aromatic moieties at the C-4 position, we wanted to investigate the effect of aryl ring torsion angle and aryl rotameric bias using 2-, 3- and 4-pyridinyl substituents[18]. Quantitative structure-activity relationships are more easily derived when small changes are gradually made on a parent compound. Pyridinyl substituents are only slightly different in surface area and dipole moment. Moreover, general structure-activity relationships, with respect to the point of attachment of the C-4 attached aryl substituents, show that optimum antagonist activity is reported according to

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[18] The results are discussed in Sections 4.2.4.1, 4.3.2.2.1 and 4.3.2.3.

the potency profile ortho ≥ meta >> para [Fossheim *et al.*, 1982; Bossert & Vater, 1989; Triggle, 1992]. It was of interest to determine whether such similar relationships also exist for pyridinyl substituents. In addition, we also wanted to do nuclear Overhauser enhancement studies to confirm the observation that 4-(2-pyridinyl) 1,4-dihydropyridines exist predominantly in the 2-pyridinyl *anti* rotameric orientation [Iqbal *et al.*, 1998; Parrish *et al.*, 2000], because such *anti* rotameric orientation induces puckering of the 1,4-dihydropyridine ring and favors drug-receptor interaction.

For our calcium channel agonist studies, a 4-benzofurazanyl or 2-trifluoromethylphenyl was attached to the C-4 position based on the activity of the classical 1,4-dihydropyridine calcium channel agonists PN 202 791 and BAY K 8644, respectively (Figure 1.4.1.3b). To compare the data with our 4-(pyridinyl) calcium channel antagonists, we decided to synthesize 4-(pyridinyl) calcium channel agonists. Additionally, the agonists prepared were to be tested for calcium channel antagonist activity on guinea pig illeum longitudinal smooth muscle. General structure-activity relationships suggest that calcium channel antagonist activity is dependent upon the size of the aryl group attached at the C-4 position [Fossheim *et al.*, 1982; Bossert & Vater, 1989; Triggle, 1992]. We also wanted to observe if such relationships existed for the groups of compounds to be investigated in this program[19].

For 1,4-dihydropyridine calcium channel antagonist activity, ester substitution on the "essential side" C-3 or C-5 position is reported as CO_2*i*-Pr > CO_2Me > COMe > CN > H [Goldmann & Stoltefuss, 1991; Triggle, 1992], which suggests that antagonist activity is proportional to the size of the substituent. We sought to further investigate this pattern by introducing 1,4-dihydropyridines possessing CO_2Et, CO_2*i*-Bu and CO_2*t-Bu* at the "essential side" C-3 position to compare with similar compound possessing CO_2*i*-Pr. Accordingly, we intended to attach a nitric oxide donor substituent at the "non-essential side" C-5 position, because this site of attachment is least likely to be detrimental to the 1,4-dihydropyridine calcium channel modulatory actions.

There are three classical 1,4-dihydropyridine calcium channel agonists. The two most potent of the three, PN 202 791 and BAY K 8644, both possess a nitro substituent at the C-3 position (Figure 1.4.1.4e and Figure 1.4.1.4f). For this reason, our proposed 1,4-dihydropyridine calcium channel agonists will also possess a C-3 nitro moiety.

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[19] The results are discussed in Section 4.3.2.3.

C-2 or C-6 substitution on the "non-essential side" has provided improved pharmacodynamic and pharmacokinetic properties with minimal loss of pharmacological activity [Romero *et al.*, 2003]. Slow onset and long acting compounds are impractical for our *in vitro* pharmacological tests. Indeed, the properties of slow onset and long duration of action are ideally tested *in vivo*. In these preliminary studies, we do not wish to profile the pharmacodynamic or pharmacokinetic properties of our compounds. All of our synthesized compounds are methyl substituted at both C-2 and C-6 positions, because methyl substituted 1,4-dihydropyridines have been reported to have good pharmacological activities and are easily made.

We maintained unsubstituted N-1 nitrogen because the proton attached to N-1 is essential for calcium channel modulation activity [Fossheim *et al.*, 1982; Fossheim, 1987; Langs *et al.*, 1991; Mager *et al.*, 1992; Triggle, 1992; Kappe & Fabian, 1997; Natale *et al.*, 1999; Zhorov *et al.*, 2001; Zamponi *et al.*, 2003].

4.1.2 NITRIC OXIDE DONORS

Of the major classes of nitric oxide donors, nitrosothiols, organic nitrates, furoxans and benzofuroxans, and N^1-substituted diazen-1-ium-1,2-diolates have been investigated as drug hybrids [Gasco *et al.*, 1996; Wang *et al.*, 2002].

Nitrosothiol preparation involves the reaction between thiol and nitric oxide derivatives such as [$\cdot NO_2$], [N_2O_4], [N_2O_3], and NO_2^- [Wang *et al.*, 2002]. Because nitrosothiols are highly unstable [Gasco *et al.*, 1996; Wang *et al.*, 2002], and because the reactants, nitrosothiols and decomposition products are malodorous, the nitrosothiol-dihydropyridine hybrids are not suitable candidates for our research.

4.1.2.1 1,4-Dihydropyridine-Organic Nitrate Hybrids

In this first project, a nitrooxy moiety was attached to an alkyl ester substituent of 1,4-dihydropyridine calcium channel antagonists[20]. In view of the facts that glyceryl trinitrate readily liberates one mole of nitric oxide with a half-life of 3 to 5 minutes to become glyceryl dinitrate [Noonan *et al.*, 1985], that glyceryl dinitrates have a half-life of approximately 40 minutes to become glyceryl mononitrates [Noonan & Benet, 1986], and that glyceryl mononitrates are therapeutically inactive [CPhA, 2003], it was expected that

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[20] The results are discussed in Sections 4.2.2.1, 4.2.4.1 and 4.3.1.2.

the extent of nitric oxide released is dependent on steric and proximity effects present in the nitrooxy moiety [Curry & Aburawi, 1985; Fung, 1992; Seth & Fung, 1993]. Nitrooxy moieties have been successfully introduced into the alkyl ester chain at the C-3 and/or C-5 position(s) of a 4-(aryl)-1,4-dihydropyridine ring system resulting in compounds possessing potent antihypertensive/calcium channel antagonist activity [Ogawa *et al.*, 1993a; Kobayashi *et al.*, 1995a; Iqbal & Knaus, 1996]. In view of these promising results, it was of interest to synthesize 4-(pyridinyl)-1,4-dihydropyridines possessing an *n*-alkyl nitrooxy moiety at the C-3 position (Figure 2a). To increase steric strain and promote nitric oxide release, a 1,3-dinitrooxy-2-propyl substituent was incorporated *in lieu* of the *n*-alkyl nitrooxy substituent. As indicated in Section 2.2, in our calcium channel antagonists, substituents were introduced on the "essential side" C-3 or C-5 ester as mid-size alkyl moieties.

Lehmann *et al.* (1997) have attached a nitrooxymethyl substituent to the 4-phenyl ring that resulted in lower vascular selectivity and potency than observed for nitrendipine and glyceryl trinitrate. In view of these disappointing results, we decided not to investigate this point of attachment unless the C-3 or C-5 substituted compounds did not exhibit useful activity.

Gasco *et al.* (1996) have also noted that the *in vitro* vasodilating activity of aliphatic nitrates is linearly dependent on lipophilicity. We wanted to observe if such relationship also exists in our group of compounds[21].

If this study provided compounds that release a high percentage of nitric oxide, it would also be of interest to synthesize a group of calcium channel agonists possessing a C-3 nitro moiety and a C-5 nitrooxy substituent with potential cardiac agonist and smooth muscle antagonist effects.

4.1.2.2 1,4-Dihydropyridine-Furoxan Hybrids

Di Stilo *et al.* (1998) and Cena *et al.* (2001) have synthesized 1,4-dihydropyridine calcium channel *antagonists* conjugated furoxanyl compounds, that possess both nitric oxide-dependent and calcium channel antagonist vasodilator activities (Figure 1.5.3f). 1,4-Dihydropyridine calcium channel agonists possessing a *4-benzofuroxanyl* moiety at the C-4 position have also been synthesized [Visentin *et al.*, 1999]. Considering the published work by other research groups, as a second project, it was of interest to attach

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[21] The results are discussed in Sections 4.2.4.1 and 4.3.1.2.

a *furoxanyl* moiety to the C-3 ester substitutent of 1,4-dihydropyridine calcium channel *agonists*, rather than the antagonists previously reported.

As discussed in Section 2.2, 4-benzofurazanyl, 2-trifluoromethylphenyl, 2-, 3- or 4-pyridinyl substituents were attached to the C-4 position[22] (Figure 2b). A nitro substituent will be present at the C-3 position to promote calcium channel agonist actions. Cena *et al.* (2001) have reported that 1,4-dihydropyridine calcium channel antagonists possessing a benzenesulfonyl or a nitrile moiety on a furoxan-4-yloxy ring release a high percentage of nitric oxide ranging from 26.8% to 40.6% (mol / mol). For this reason, we were interested in nitric oxide donor 3-(benzenesulfonyl)furoxan-4-yloxy substituents. If the initial pharmacological results were encouraging, the design of compounds that exhibit dual cardioselective positive inotropic / smooth muscle relaxant effect would be warranted.

4.1.2.3 1,4-Dihydropyridine-(N^1-Substituted Diazen-1-ium-1,2-diolate) Hybrids

No other research group has attached an N^1-substituted diazen-1-ium-1,2-diolate moiety to a 1,4-dihydropyridine calcium channel agonist or antagonist.

The synthesis of 1,4-dihydropyridine antagonists possessing an O^2-sodium salt of 1-(*N*-substituted)diazen-1-ium-1,2-diolate moiety was attempted. Regrettably, during the final step of synthesis, these hybrid compounds either cleaved into the two parent compounds, or decomposed by releasing nitric oxide (Section 4.2.2.3).

A group of 1,4-dihydropyridine agonists possessing an O^2-alkyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate moiety has been synthesized [Velázquez *et al.*, 2003] (Figure 1.5.4e). Unfortunately, these compounds did not release nitric oxide in the absence or presence of the thiol L-cysteine, pig liver esterase or rat serum.

Subsequently, a group of 1,4-dihydropyridine calcium channel agonists possessing an O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino, or 4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate moiety was successfully synthesized [Velázquez & Knaus, 2004] (Figure 1.5.4e). Although these compounds exhibited weak smooth muscle calcium channel antagonist (relaxation) and weak cardiac calcium channel agonist (positive inotropy) activity, they released a substantial amount of nitric oxide ranging from 121% to 170% (mol / mol) in a solution of guinea pig serum and phosphate buffer.

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[22] The results are discussed in Sections 4.2.2.2, 4.2.4.2 and 4.3.1.3.

Moreover, the adverse effect of smooth muscle contraction was not observed in any of the compounds.

The objective of this research program was to synthesize 1,4-dihydropyridine calcium channel antagonists possessing an O^2-acetoxymethyl-1-(*N*-ethyl-*N*-alkylamino, or 4-ethylpiperazin-1-yl)diazen-1-ium-1,2-diolate moiety[23] (Figure 2c). As discussed in Section 2.2, in our calcium channel antagonists, the strategy was to position substituents on the "essential side" C-3 or C-5 ester as mid-size alkyl moieties.

It was also of interest to vary the *N*-alkylamino portion of the N^1-substituted diazen-1-ium-1,2-diolate to seek a correlation with the extent of nitric oxide released[24] [Hrabie & Keefer, 2002]. Mu *et al.* (2000) have reported a nicorandil-furoxan hybrid that exhibit a gradual and sustained antihypertensive effect in anaesthetized rats (Figure 1.5.3d). That nicorandil-furoxan hybrid possesses a piperazinyl moiety. Additionally, Meguro *et al.* (1985) reported piperazinylalkyl ester 1,4-dihydropyridine calcium channel antagonists that exhibited a potent and long-lasting antihypertensive effect. In view of these promising results, the 4-ethylpiperazin-1-yl moiety was used as an anchor point for the N^1-substituted diazen-1-ium-1,2-diolate.

Pending encouraging pharmacological results, the attachment of an aminoalkoxy substituent at either the C-2 or C-6 position of the 1,4-dihydropyridine moiety to mimic the slow onset and long duration of calcium channel antagonist effects, like amlodipine, would also be pursued [Romero *et al.*, 2003] (Figure 1.4.1.5b). This aminoalkoxy substituent may serve as a point of attachment for an N^1-substituted diazen-1-ium-1,2-diolate. In simpler words, a 1,4-dihydropyridine-(N^1-substituted diazen-1-ium-1,2-diolate) hybrid is a prodrug that, upon release of nitric oxide, may share a similar calcium channel antagonist profile with amlodipine. Moreover, one could synthesize 1,4-dihydropyridines possessing two N^1-substituted diazen-1-ium-1,2-diolate moieties on the "non-essential side" at the C-2 and C-3 positions.

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[23] The results are discussed in Section 4.2.4.3.

[24] The results are discussed in Sections 4.3.1.5 and 4.3.1.6.

4.1.3 PHARMACOLOGY

4.1.3.1 Measurement of Calcium Channel Modulatory Activities

Because calcium channel modulators affect the entire cardiovascular system and respond to different stimuli, various experimental protocols are required to test for calcium channel agonist and calcium channel antagonist activities. Procedures that are cost-effective with minimal experimental errors are most desirable for the acquisition of structure-activity data.

Calcium channel antagonists are frequently potent vasodilators due to their smooth muscle relaxant effect. Because vascular tissues are small and difficult to handle, another type of smooth muscle, guinea pig ileum longitiudinal smooth muscle, was used instead. Measuring calcium channel antagonist activity requires a stimulus to contract the muscle. This stimulus can either be chemical or electrical. Because calcium channel modulators are known to switch pharmacological profile at different voltage [Sanguinetti & Kass, 1984a; Sanguinetti & Kass, 1984b], a chemical stimulus, such as the muscarinic receptor agonist, carbachol, is more appropriate.

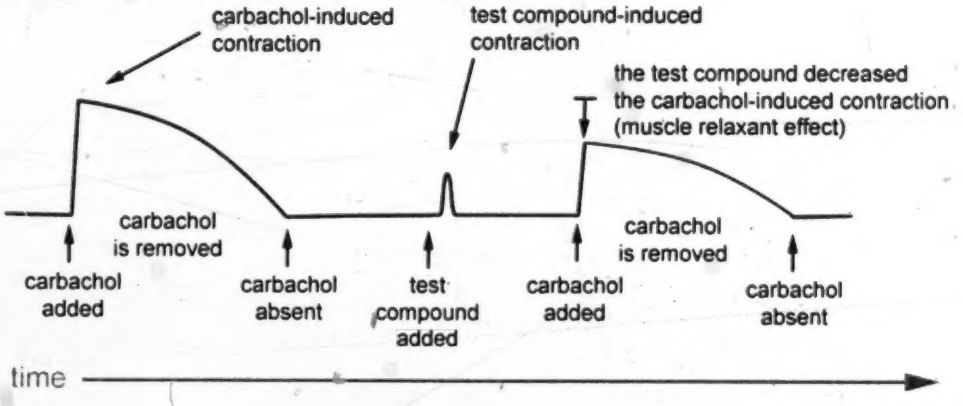

Figure 4.1.3.1a: A Polygraph Schematic Showing the Effects of a 1,4-Dihydropyridine Calcium Channel Modulator Racemate Possessing Smooth Muscle Contraction and Relaxation Properties on a Guinea Pig Ileum Longitudinal Smooth Muscle

The simplest model is to suspend the guinea pig ileum longitudinal smooth muscle in an oxygenated buffer solution in an organ bath, then induce a muscular contraction by adding carbachol to the solution (Figure 4.1.3.1a). The tissue contractile

force is measured with a force-displacement transducer. After replacing the carbachol containing solution with fresh buffer solution and allowing the tissue to re-equilibrate, the test compound is added and left to perfuse into the tissue. After 5 minutes, carbachol is added to the solution to stimulate a muscular contraction. The presence of a test compound with calcium channel antagonist activity exhibits a weaker carbachol-induced muscular contraction relative to the carbachol-induced contraction in the absence of the test compound. Indeed, smooth muscle calcium channel antagonist activity is determined as the molar concentration of the test compound required to produce 50% inhibition of the muscarinic receptor-mediated (carbachol, 0.167 μM) calcium-dependent contraction (tonic response) of guinea pig ileum longitudinal smooth muscle [Vo *et al.*, 1995]. A tissue sample's IC_{50} value was determined graphically, when possible, from the dose-response curve. The average of all samples' IC_{50} values represented the test compound's smooth muscle antagonistic IC_{50} value (± SEM, n = 2 or 3).

Interestingly, the undesired effect of smooth muscle contraction induced by the test compound can also be measured. The test compound is a racemate. If one of the enantiomers of the test compound has smooth muscle contractile properties, a contraction is induced when the test compound is added to the solution (Figure 4.1.3.1a). Calcium channel receptor-mediated contractions are not sustained as muscarinic receptor-mediated contractions. Calcium channel agonist (contractile) activity on guinea pig ileum longitudinal smooth muscle (in the absence of 0.167 μM carbachol) is calculated as the molar concentration of the test compound required to elicit a 50% increase in the contractile response, relative to the response produced by carbachol (0.167 μM).). A tissue sample's EC_{50} value was determined graphically, when possible, from the dose-response curve. The average of three samples' EC_{50} values represented the test compound's smooth muscle agonistic EC_{50} value (± SEM, n = 3).

On a side-note, non-cardioselective calcium channel antagonists also have a role on the drug market. Indeed, the calcium channel antagonist, pinaverium bromide, is used for the symptomatic treatment of abdominal pain, bowel disturbances and intestinal discomfort related to irritable bowel syndrome [CPhA, 2003] (Table 1.2.2). Patients with irritable bowel syndrome have hypersensitive nerves that cause the bowel to contract abnormally, resulting in pain, bloatedness, diarrhea and constipation.

In this research program, guinea pig ileum longitudinal muscle was used to test for calcium channel antagonist activity. For the groups of calcium channel antagonists

investigated, a potent antagonist should induce a significant response in the ileum, and hence, can also be used as symptomatic treatment for irritable bowel syndrome. For the groups of calcium channel agonist described in this thesis, a cardioselective agonist should ideally have minimal smooth muscle contraction effects on the ileum[25].

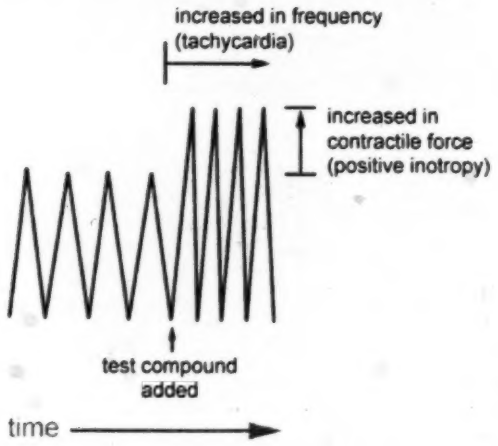

Figure 4.1.3.1b: A Polygraph Schematic Showing the Effect of a 1,4-Dihydropyridine Calcium Channel Agonist Possessing Positive Inotropic and Tachycardic Properties on a Guinea Pig Left Atrium

The key beneficial action of calcium channel agonists is their ability to increase cardiac contractile force without increasing heart rate. The simplest model is to suspend a guinea pig atrium in an oxygenated buffer solution using an organ bath, while electrically stimulating the tissue, add the test compound into the solution, and observe changes in contractile force and heart rate [Vo *et al.*, 1995] (Figure 4.1.3.1b). Because the left atrium is a larger muscle than the right atrium, the right atrium is used to study chronotropic effects, and the left atrium to study inotropic effects. The cardiac calcium channel agonist effect is calculated as the percentage increase (positive inotropic effect) in contractile force of isolated guinea pig left atrium relative to its basal contractile force in the absence of the test compound. A tissue sample's EC_{50} value was determined graphically, when possible, from the dose-response curve. The average of three samples' EC_{50} values represented the test compound's cardiac positive inotropic EC_{50}

[25] The results are discussed in Sections 4.3.1.3, 4.3.2.2 and 4.3.2.2.2.

value (± SEM, n = 3). The change in heart rate is calculated as the percentage increase in beats per second of isolated guinea pig right atrium relative to its basal heart rate in the absence of the test compound. Measurements are performed with a force-displacement transducer and recorded on a polygraph machine. The amplitude of each peak determines contractile force, while the number of peaks per time interval determines the heart rate.

The *in vitro* pharmacological testing of the 1,4-dihydropyridine calcium channel modulators investigated were tested as racemates. In the case of compounds possessing a 1,4-dihydropyridine C-3 nitro substituent, one enantiomer may exhibit agonist properties while the other enantiomer may exhibit antagonist properties [Mahmoudian & Richards, 1986a; Triggle & Rampe, 1989; Triggle, 1992]. The 1,4-dihydropyridine agonists were monitored for tachycardia[26].

4.1.3.2 Indirect Measurement of Percent Nitric Oxide Release

A 1,4-dihydropyridine calcium channel modulator possessing a nitric oxide donor moiety should release nitric oxide. When tested under the same environmental conditions, the extent of nitric oxide released depends on the nature of the compound. Compounds releasing large quantities of nitric oxide are strong vasodilators that may potentiate the desired cardiovascular actions of the 1,4-dihydropyridine calcium channel modulators.

Privat *et al.* (1997) reviewed three commonly used methods to quantify nitric oxide productions, namely the Hemoglobin Reaction, Electrochemical Detection and Griess Reaction methods.

$$HbO_2 + \dot{N}O \longrightarrow MetHb + NO_3^-$$

Figure 4.1.3.2a: Oxyhemoglobin Reacts with Nitric Oxide to Form Methemoglobin

The Hemoglobin Reaction method is based on the quantitative oxidation of oxyhemoglobin Fe(II) to methemoglobin Fe(III) in aqueous solution by nitric oxide (Figure 4.1.3.2a). At pH 7.4, methoxyhemoglobin has a characteristic absorption at 405

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[26] The results are discussed in Section 4.3.2.2.

nm. A mixture of oxyhemoglobin and methemoglobin exhibits an isobestic point[27] at 411 nm. Accordingly, this method does not directly measure nitric oxide release. Nitric oxide release is predicted by comparing the absorbances at 405 nm (methemoglobin) and 411 nm (oxyhemoglobin) either as ($A_{405} - A_{411}$) or (A_{405} / A_{411}) relative to a standard methemoglobin-oxyhemoglobin concentration curve.

Regrettably, the Hemoglobin Reaction method is not reproducible in that small changes in time interval, pH and temperature can greatly influence the hemoglobin reaction. In addition, oxyhemoglobin has a very high affinity for nitric oxide. This rapid trapping can influence nitric oxide release. Thus, the assay itself may affect nitric oxide production rate.

The Electrochemical Detection method is based on the direct measurement of the nitric oxide oxidation current by differential pulse amperometry at 0.75 V versus Ag / AgCl. In simpler words, nitric oxide is detected and quantified by an electrode probe. Of the three methods used to predict nitric oxide release, this technique is the only one for *in situ*, direct, specific, non-destructive and instantaneous measurements of nitric oxide concentrations. This technique is also the only method that permits easy kinetic studies of nitric oxide production. However, the electrode has a relatively short lifetime of approximately 10 hours.

$$2\,\dot{N}O + O_2 \longrightarrow \cdots \xrightarrow{H^+} 2\,HNO_2$$

$$\xrightarrow{H^+} HNO_2 + HNO_3$$

Figure 4.1.3.2b: Nitric Oxide Reacts in an Aerobic Acidic Aqueous Solution to Form Nitrous Acid and Nitric Acid

The Griess Reaction method determines nitrite concentration. Evidently, this method does not directly quantify nitric oxide release, but measures instead the product of nitric oxide decomposition (Section 1.5.6). In an aerobic acidic aqueous solution, nitrites are protonated to form nitrous acids (Figure 4.1.3.2b). Nitrous acids react with sulfanilamide to produce a diazoic compound (Figure 4.1.3.2c) that reacts with *N*-(1-

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[27] The wavelength at which the specific absorbances of two interconvertible materials are the same, regardless of the equilibrium position of the reaction between them

naphthyl)ethylenediamine to form a purple azo dye (Figure 4.1.3.2d). Because the absorbance of the azo dye at 540 nm is directly proportional to the nitrite content of the standard solution, nitrite determination is based on a sodium nitrite concentration curve.

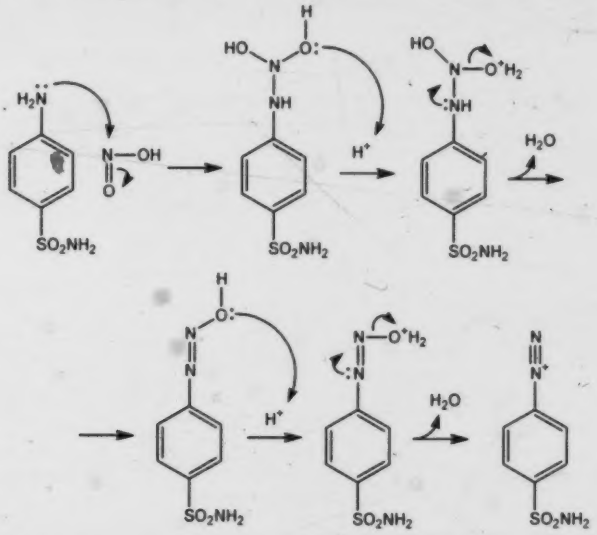

Figure 4.1.3.2c: Nitrous Acid Reacts with Sulfanilic Acid to Form a Diazoic Compound

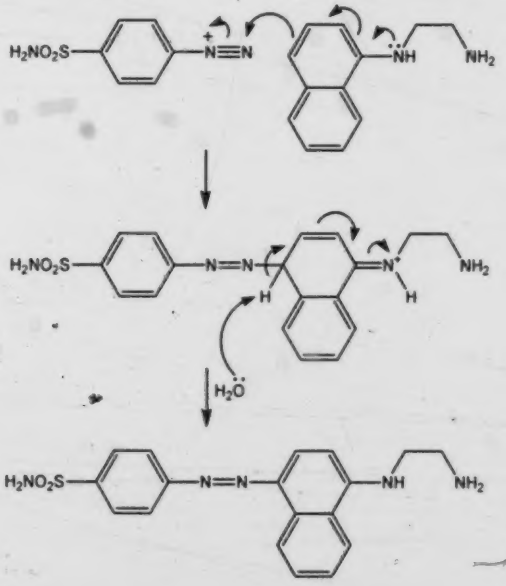

Figure 4.1.3.2d: The Diazoic Compound Reacts with *N*-(1-Naphthyl)ethylenediamine to Form a Purple Azo Dye

With the Griess Reaction method, nitrate concentration can also be measured. Nitrate needs to be reduced to nitrite in order to be quantifiable. Nitrate reduction is an enzymatic process involving NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nitrate reductase. However, because nitrite production strictly follows nitric oxide production with a 99.8% correlation, accounting nitrate production as a predictor of nitric oxide released often leads to an over-estimation.

The Griess Reaction method was used in this research program to measure nitrite that arose from released nitric oxide. Nitrate was not considered in the calculations in order to avoid over-estimations. The Hemoglobin Reaction method is prone to unacceptable experimental errors leading to results that are not reproducible, while the Electrochemical Detection method is not cost-effective.

4.1.3.3 Analyses of Pharmacological Results

It is of interest to correlate chemical structure with pharmacological activity, based on the pharmacological results of the synthesized compounds, namely positive inotropy in guinea pig left atrium, guinea pig ileum longitudinal smooth muscle contraction or relaxation, and the extent of nitric oxide released.

Tachycardia and smooth muscle contractions are undesired pharmacological effects. We expect the compounds investigated to have minimal tachycardia and smooth muscle contraction effects.

Attempts will be made to derive multiple collinear regression equations using the Property-Intrinsic Activity Relationships method[28] (Section 1.4.1). We suspect that calcium channel modulatory actions are complicated. On the other hand, we believe that the extent of nitric oxide released shares simple relationships with structural properties.

The Zhorov *et al.* (2001) and Zamponi *et al.* (2003) research groups have modified the potassium channel coordinates in an attempt to apply their models to the calcium channel protein. We will test the two models with the classical calcium channel antagonist, nifedipine, and the classical calcium channel agonist, BAY K 8644[29]. We will

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[28] The results are discussed in Sections 4.3.1 and 4.3.2.

[29] The results are discussed in Section 4.3.3.

also do computer graphics simulated docking of our compounds into the receptors in an attempt to correlate energy of stabilization with pharmacological activity.

4.2 SYNTHESIS OF 1,4-DIHYDROPYRIDINE CALCIUM CHANNEL MODULATORS

4.2.1 Synthesis of the 1,4-Dihydropyridine Pharmacophore

This section (4.2.1) of the thesis will discuss the synthesis of the basic 1,4-dihydropyridine ring.

There are three common methods of preparing the central 1,4-dihydropyridine ring. Each method is named accordingly to the number of reaction steps that are required to condense the starting materials.

4.2.1.1 One-Step Hantzsch Condensation Method

A. Hantzsch first synthesized 1,4-dihydropyridines in 1882 from two alkyl acetoacetates, an aryl aldehyde and ammonia [Goldmann & Stoltefuss, 1991] (Figure 4.2.1.1). This classical Hantzsch condensation method also produced two symmetrical 1,4-dihydropyridines along with the desired product,.

The Hantzsch condensation occurs in several stages [Bossert & Vater, 1989], thus synthesis can be performed using a one-, two-, or three-step method [Meguro *et al.*, 1985; Yiu & Knaus, 1996]. The selection of the appropriate method is dependent on the stability and ease of purification of the intermediate products (Sections 4.2.1.2 and 4.2.1.3).

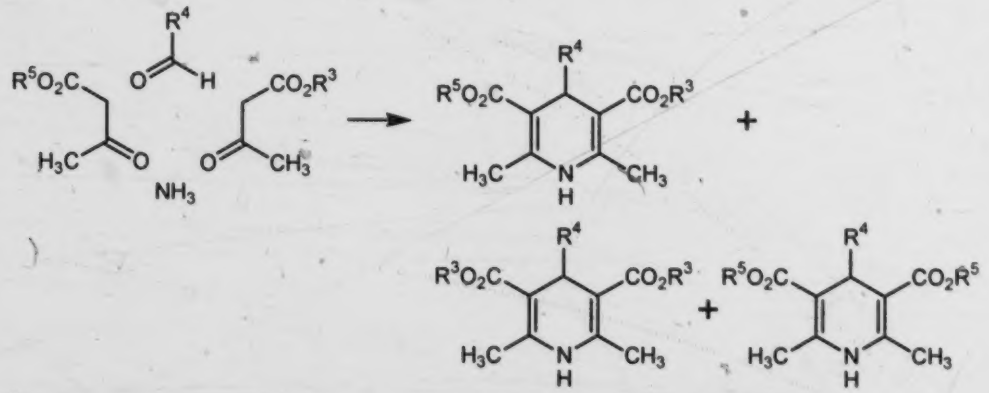

Figure 4.2.1.1: One-Step Hantzsch Condensation Method

4.2.1.2 Two-Step Hantzsch Condensation Method

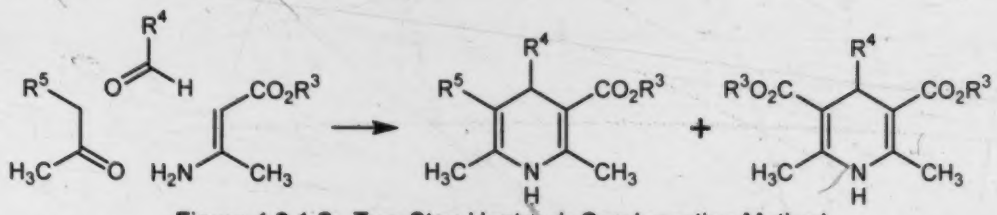

Figure 4.2.1.2: Two-Step Hantzsch Condensation Method

The most commonly used method involves the condensation of an aryl aldehyde, 3-alkylpropan-2-one and alkyl 3-aminocrotonate (Figure 4.2.1.2).

4.2.1.2.1 Synthesis of 3-Alkylpropan-2-ones

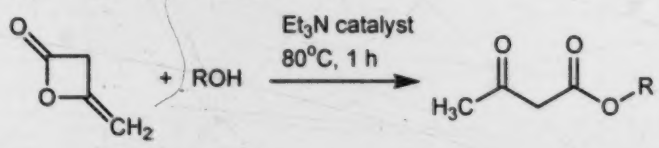

Figure 4.2.1.2.1a: General Synthesis of Alkyl Acetoacetates Using Diketene

When 1,4-dihydropyridine-3,5-dicarboxylic acid diester calcium channel antagonists are synthesized, the 3-alkylpropan-2-one equivalent reagent is actually an alkyl acetoacetate. The "alkyl acetoacetate" is also traditionally referred to as an "acetyl acetatetic ester" [Goldmann & Stoltefuss, 1991] or "β-ketocarboxyl acid ester" [Bossert & Vater, 1989]. The traditional method of preparing an alkyl acetoacetate is achieved by reacting an alcohol with diketene (Figure 4.2.1.2.1a). A diketene is a highly reactive, lachrymator[30] and toxic reagent. Clemens and Hyatt (1985) showed that pyrolysis of 2,2,6-trimethyl-4*H*-dioxin-4-one in the presence of a nucleophile, such as an alcohol, provided an excellent "general-purpose" acetoacetylation method providing good yields (Figure 4.2.1.2.1b). 2,2,6-Trimethyl-4*H*-dioxin-4-one is a weaker lachrymator, more stable, less hazardous and more economical than diketene. Reaction with the dioxinone requires no catalyst and provides mainly volatile by-products. I have utilized both

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[30] A tear-producing substance

methods and found that diketene reactions afford slightly higher product yield and purity than the dioxinone reactions. However, the dioxinone procedure is often preferable because diketene has a short shelf-life, induces nausea and is a potent lachrymator.

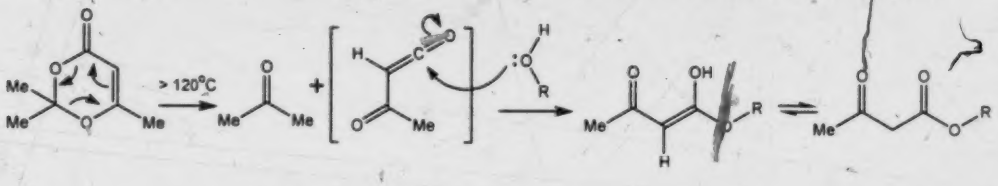


Figure 4.2.1.2.1b: General Synthesis of Alkyl Acetoacetates Using 2,2,6-Trimethyl-4*H*-dioxin-4-one

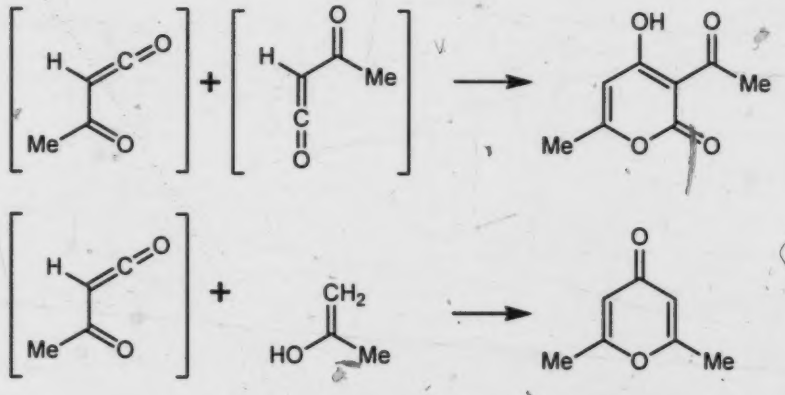


Figure 4.2.1.2.1c: By-Products of the Dioxinone Reaction

Clemens and Hyatt (1985) identified two major by-products (Figure 4.2.1.2.1c). When heated to 120 – 150°C in the absence of a nucleophile, the dioxinone formed a dehydroacetic acid via (4 + 2) cycloaddition of two acetylketenes. Moreover, an acetylketene in the presence of excess acetone increased the amount of pyrone formed. In my studies, the by-products were not purified, and I cannot confirm the presence of these by-products.

Clemens and Hyatt (1985) suggested that the dioxinone reaction be done under reflux in xylene for 30 minutes (compounds **2a-e**) (Figure 4.2.1.2.1d). In preparing compounds **2a-e**, **40** and 2-cyanoethyl acetoacetate, a similar procedure was used except that the reaction progress was monitored by TLC. A longer reaction time resulted in higher product yields.

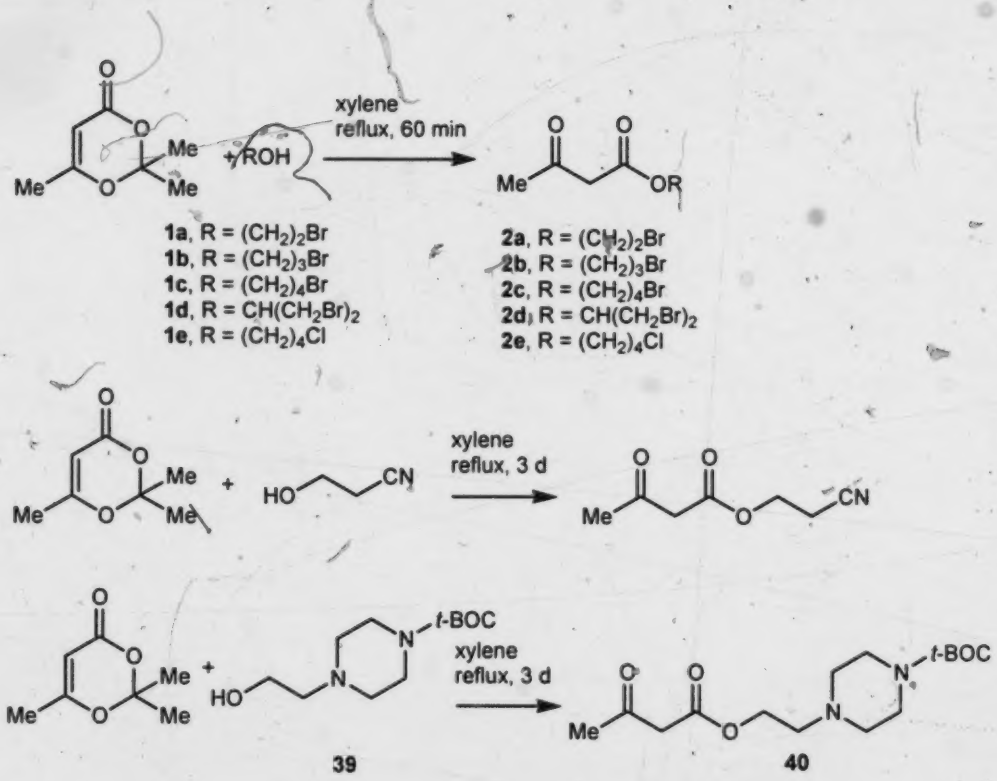
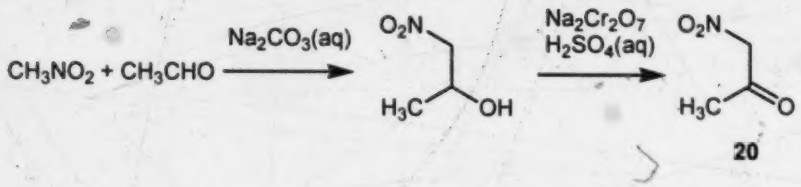


Figure 4.2.1.2.1d: Synthesis of Alkyl Acetoacetates (**2a-e** and **40**)

In the case of 1,4-dihydro-3-nitropyridine calcium channel agonists, the 3-alkylpropan-2-one equivalent reagent is actually nitroacetone (**20**). The preparation of nitroacetone (**20**) is a classical method that has undergone very little improvements since 1955 [Hurd & Nilson, 1955] (Figure 4.2.1.2.1e). The method involves the formation of 1-nitro-2-propanol by reacting nitromethane with acetaldehyde in a basic aqueous solution at 22°C for 6.5 hours. 1-Nitro-2-propanol was purified by vacuum distillation. It was found that the 1-nitro-2-propanol mixture is fairly explosive upon heating. 1-Nitro-2-propanol is oxidized by chromic acid (H_2CrO_4), which is prepared *in situ* from sodium dichromate ($Na_2Cr_2O_7$) and sulfuric acid (H_2SO_4), at 10 – 20°C for 6 hours to form nitroacetone crystals (**20**) that are then purified by re-crystallization. It was observed that the yield of nitroacetone (**20**) is often lower than that which was reported by Hurd and Nilson (1955). Nitroacetone (**20**) is highly unstable and can only be stored for a short period of time under diethyl ether at -5°C.



20

Figure 4.2.1.2.1e: Synthesis of Nitroacetone (**20**)

4.2.1.2.2 Synthesis of Alkyl 3-Aminocrotonates

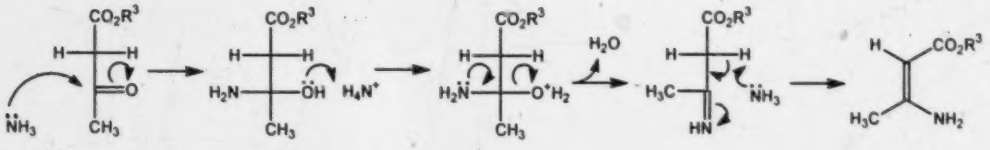


Figure 4.2.1.2.2a: General Synthesis of Alkyl 3-Aminocrotonates

Alkyl 3-aminocrotonates are synthesized by bubbling ammonia gas through a solution of an alkyl acetoacetate in either dichloromethane or diethyl ether depending on the solubility of the starting material (Figure 4.2.1.2.2a). The product often precipitates from diethyl ether. The reaction is facile often requiring less than 30 minutes to obtain a yield from 90% to 100% (Figure 4.2.1.2.2b). 2-Cyanoethyl acetoacetate is slightly less reactive and requires up to 20 hours for the reaction to form a solid, 2-cyanoethyl 3-aminocrotonate (**21**) that tends to clog the ammonia inlet valve. Hence, the reaction was carried out in dichloromethane in which the product was soluble. Similarly, the preparation of 2-(4-[*t*-butoxycarbonyl]piperazinyl)ethyl 3-aminocrotonate (**41**) requires 9 hours for the reaction to complete..

If the reaction to prepare the alkyl 3-aminocrotonate does not proceed at an acceptable rate, an alternative method is available [Reeve *et al.*, 1992]. A mixture of ammonium acetate (25 mmol) and alkyl acetoacetate (13.2 mmol) in dry toluene (50 mL) is refluxed for 3.5 hours with azeotropic removal of water. The reaction mixture is then cooled, washed with a saturated solution of sodium bicarbonate, and dried over anhydrous sodium sulfate. Removal of the solvent *in vacuo* affords the desired product. Comparatively, this ammonium acetate method requires more purification than the ammonia method.

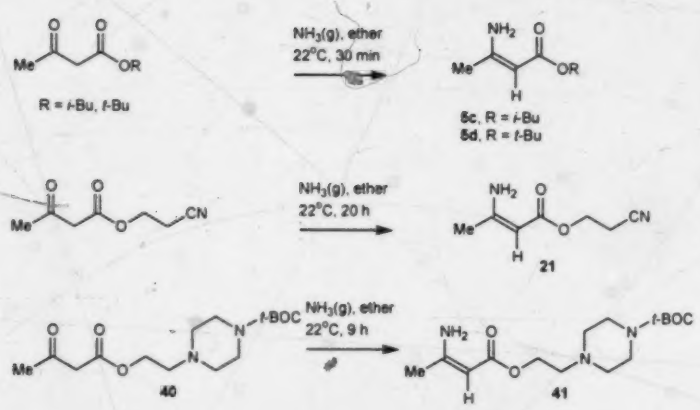


Figure 4.2.1.2.2b: Synthesis of Alkyl 3-Aminocrotonates (**5c-d**, **40**, **41**)

4.2.1.2.3 Hantzsch Condensation of an Aryl Aldehyde, 3-Alkylprop-2-one and Alkyl 3-Aminocrotonate

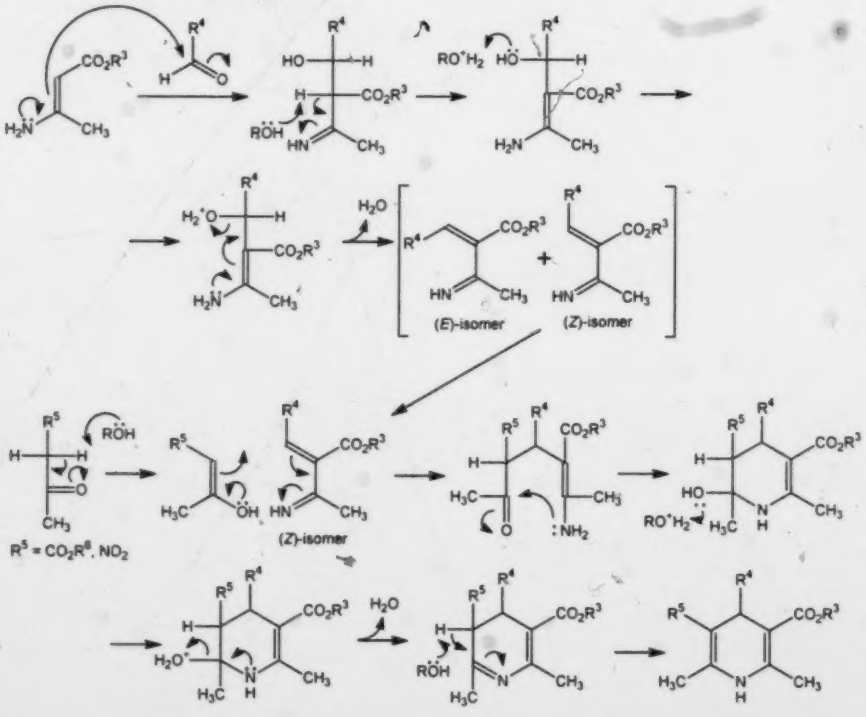


Figure 4.2.1.2.3a: Hantzsch Condensation of an Aryl Aldehyde, 3-Alkylprop-2-one and Alkyl 3-Aminocrotonate

The alkyl 3-amincrotonate reacts with an aryl aldehyde, then cyclizes upon reaction with a 3-alkylpropan-2-one to form a Hantzsch 1,4-dihydropyridine (Figure 4.2.1.2.3a). Depending on the solubility of the reagents, the reaction is performed in either ethanol or isopropanol. Moreover, depending on the reactivity, selectivity and stability of the reaction, the reaction time varies from 1 to 3 days, and the reaction temperature ranges from 22°C to 80°C.

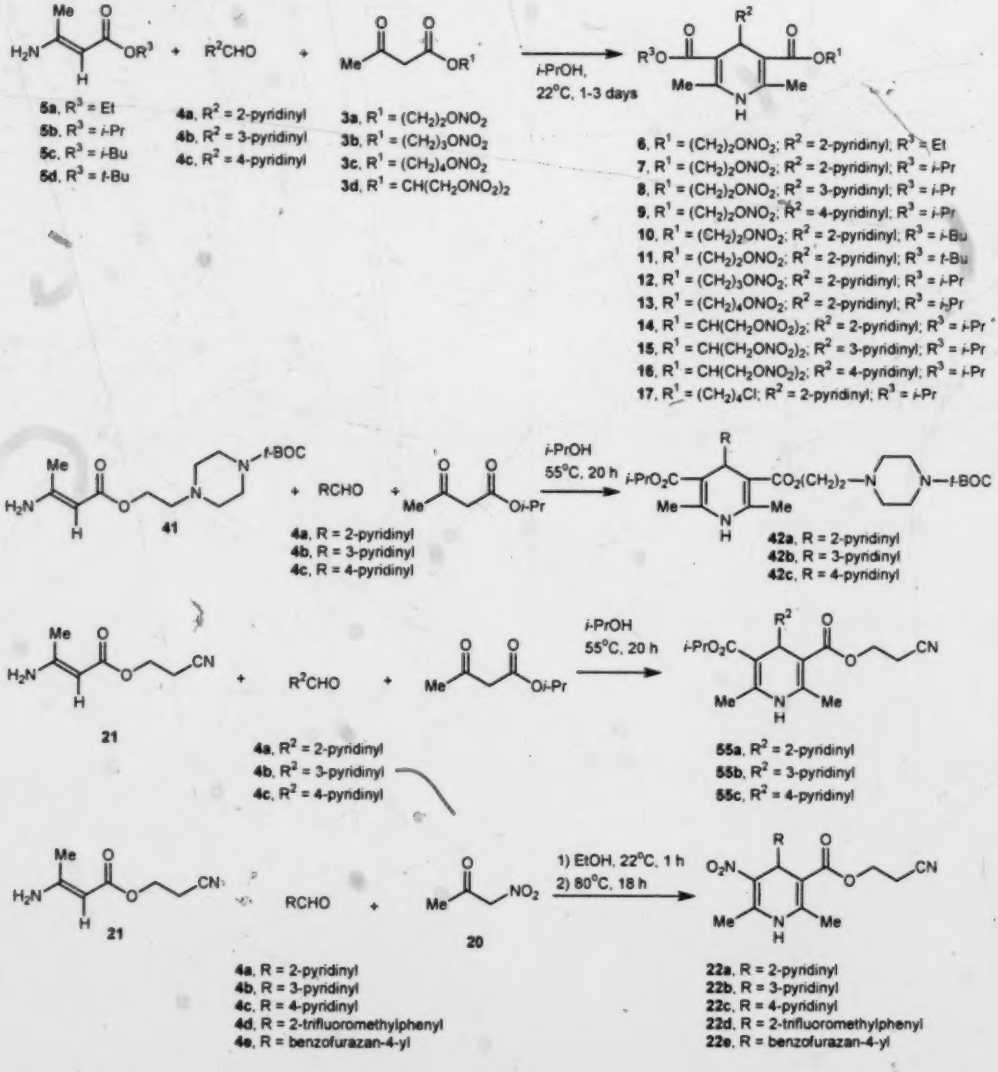

5a, R3 = Et
5b, R3 = i-Pr
5c, R3 = i-Bu
5d, R3 = t-Bu
4a, R2 = 2-pyridinyl
4b, R2 = 3-pyridinyl
4c, R2 = 4-pyridinyl
3a, R1 = (CH2)2ONO2
3b, R1 = (CH2)3ONO2
3c, R1 = (CH2)4ONO2
3d, R1 = CH(CH2ONO2)2
i-PrOH, 22°C, 1-3 days
6, R1 = (CH2)2ONO2; R2 = 2-pyridinyl; R3 = Et
7, R1 = (CH2)2ONO2; R2 = 2-pyridinyl; R3 = i-Pr
8, R1 = (CH2)2ONO2; R2 = 3-pyridinyl; R3 = i-Pr
9, R1 = (CH2)2ONO2; R2 = 4-pyridinyl; R3 = i-Pr
10, R1 = (CH2)2ONO2; R2 = 2-pyridinyl; R3 = i-Bu
11, R1 = (CH2)2ONO2; R2 = 2-pyridinyl; R3 = t-Bu
12, R1 = (CH2)3ONO2; R2 = 2-pyridinyl; R3 = i-Pr
13, R1 = (CH2)4ONO2; R2 = 2-pyridinyl; R3 = i-Pr
14, R1 = CH(CH2ONO2)2; R2 = 2-pyridinyl; R3 = i-Pr
15, R1 = CH(CH2ONO2)2; R2 = 3-pyridinyl; R3 = i-Pr
16, R1 = CH(CH2ONO2)2; R2 = 4-pyridinyl; R3 = i-Pr
17, R1 = (CH2)4Cl; R2 = 2-pyridinyl; R3 = i-Pr
41
4a, R = 2-pyridinyl
4b, R = 3-pyridinyl
4c, R = 4-pyridinyl
i-PrOH 55°C, 20 h
42a, R = 2-pyridinyl
42b, R = 3-pyridinyl
42c, R = 4-pyridinyl
21
4a, R2 = 2-pyridinyl
4b, R2 = 3-pyridinyl
4c, R2 = 4-pyridinyl
i-PrOH 55°C, 20 h
55a, R2 = 2-pyridinyl
55b, R2 = 3-pyridinyl
55c, R2 = 4-pyridinyl
21
20
1) EtOH, 22°C, 1 h
2) 80°C, 18 h
4a, R = 2-pyridinyl
4b, R = 3-pyridinyl
4c, R = 4-pyridinyl
4d, R = 2-trifluoromethylphenyl
4e, R = benzofurazan-4-yl
22a, R = 2-pyridinyl
22b, R = 3-pyridinyl
22c, R = 4-pyridinyl
22d, R = 2-trifluoromethylphenyl
22e, R = benzofurazan-4-yl

Figure 4.2.1.2.3b: Synthesis of Hantzsch 1,4-Dihydropyridines (**6-17**, **42a-c**, **22a-e** and **55a-c**) Using the Two-Step Hantzsch Reaction

Most literature reports list a reaction time of 20 hours at 80°C [Meguro *et al.*, 1985; Baldwin *et al.*, 1987; Dagnino *et al.*, 1987a; Ramesh *et al.*, 1992; Kawashima *et al.*, 1993; Kobayashi *et al.*, 1995a; Kobayashi *et al.*, 1995b; Vo *et al.*, 1995; Iqbal & Knaus, 1996; Yiu & Knaus, 1996; Lehman *et al.*, 1997; Yiu and Knaus, 1997; Iqbal *et al.*, 1998; Di Stilo *et al.*, 1998; Natale *et al.*, 1999; Ramesh *et al.*, 1999; Shan & Knaus, 1999; Visentin *et al.*, 1999; Miri *et al.*, 2000; Amini *et al.*, 2001; Shan *et al.*, 2002; Fassihi *et al.*, 2004].

In these studies to prepare calcium channel antagonists (**6-17**, **42a-c** and **55a-c**), it was found that a lower reaction temperature of 45 – 55°C for 20 hours provided an acceptable reaction time while minimizing the number of by-products (Figure 4.2.1.2.3b). In the case of 1,4-dihydro-3-nitropyridine calcium channel agonists (**22a-e**), the starting material nitroacetone (**20**) is unstable and insoluble in isopropanol. Consequently, 1,4-dihydro-3-nitropyridine calcium channel agonists are best prepared using ethanol as solvent at 55 – 60°C for 18 hours. Nitroacetone remains in solution using these reaction conditions. If reaction time exceeds 20 hours, nitroacetone (**20**) decomposes and its purification becomes much more difficult.

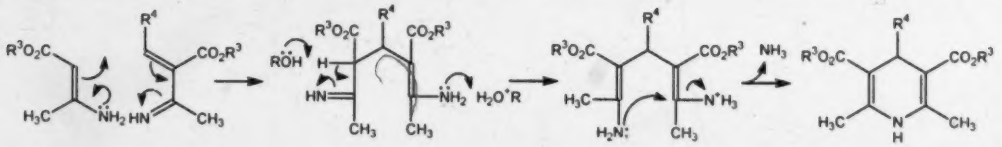


Figure 4.2.1.2.3c: Formation of the First Generation Symmetrical 1,4-Dihydropyridine using the Two-Step Hantzsch Condensation Method

Symmetrical 1,4-dihydropyridine by-products are also formed in this Two-Step Hantzsch reaction (Figure 4.2.1.2.3c). The symmetrical 1,4-dihydropyridine can be avoided if (*Z*)-2-arylidene-3-iminobutyric acid alkyl ester is isolated prior to adding 3-alkylpropan-2-one, or by following the third method for modifying the Hantzsch condensation (Section 4.2.1.3).

Moreover, ammonia that is released (Figure 4.2.1.2.3c) reacts with 3-alkylpropan-2-one. Consequently, small quantities of a second symmetrical 1,4-dihydropyridine are produced (Figure 4.2.1.2.3d).

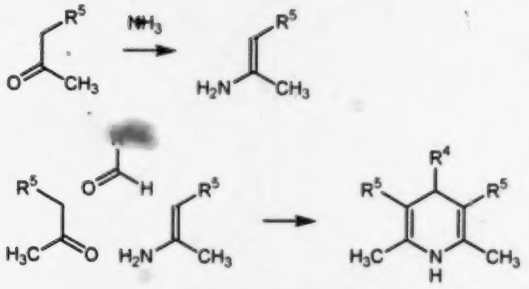


Figure 4.2.1.2.3d: Formation of the Second Generation Symmetrical 1,4-Dihydropyridine using the Two-Step Hantzsch Condensation Method

4.2.1.3 Three-Step Hantzsch Condensation Method

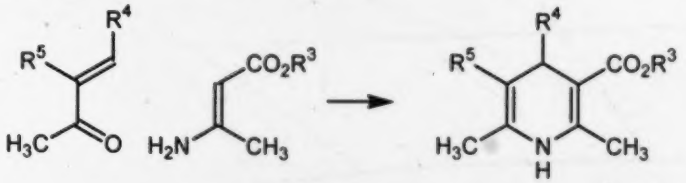


Figure 4.2.1.3: Three-Step Hantzsch Condensation Method

A modified Hantzsch condensation, in which an alkyl 3-aminocrotonate is combined with a (*Z*)-3-alkyl-4-arylbut-3-en-2-one (assuming that the R^4 and R^5 have higher name-ranking priorities), can simplify the separation and purification of the final 1,4-dihydropyridine (Figure 4.2.1.3) [Yiu & Knaus, 1996].

4.2.1.3.1 Synthesis of (*Z*)-3-Alkyl-4-arylbut-3-en-2-ones

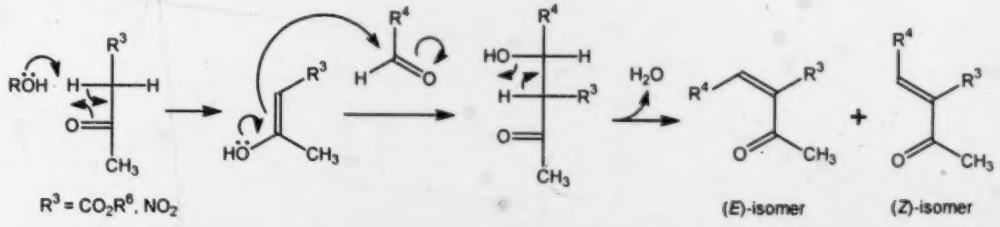


Figure 4.2.1.3.1: General Synthesis of (*Z*)-3-Alkyl-4-arylbut-3-en-2-ones

Similar to the reaction conditions used during a Hantzsch condensation, (*Z*)-3-alkyl-4-arylbut-3-en-2-one (assuming that the R^3 and R^4 have higher name-ranking priorities) is synthesized by condensing a 3-alkylpropan-2-one and an aryl adehyde at 45 – 55°C for 20 hours (Figure 4.2.1.3.1). Separation of the isolation of the (*Z*)- from (*E*)-geometric isomers is often difficult. In the case of the pyridinyl agonists (**24-32**) (Section 3.1.2), (*Z*)-3-nitro-4-(pyridinyl)but-3-en-2-ones are highly unstable. Hence, the Two-Step Hantzsch Condensation method was used (Sections 3.1.2.2 and 4.2.1.2).

4.2.1.3.2 Hantzsch Condensation of (*Z*)-3-Alkyl-4-arylbut-3-en-2-one and a 2-Amino-3-alkylprop-2-ene

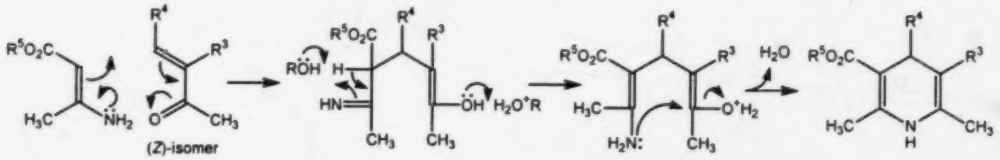


Figure 4.2.1.3.2: Hantzsch Condensation of a (*Z*)-3-Alkyl-4-arylbut-3-en-2-one and an Alkyl 3-Aminocrotonate

The condensation of an alkyl 3-amincrotonate with a (*Z*)-3-alkyl-4-arylbut-3-en-2-one at 45 – 55°C for 20 hours, usually yields an asymmetrical 1,4-dihydropyridine product that is easier to purify than the same product prepared using a Two-Step Hantzsch Condensation method (Figure 4.2.1.3.2). However, the decision to use the Two- or Three-Step Hantzsch Condensation method should also consider the nature of the reactants, products and reaction conditions.

4.2.1.4 Choosing Which Side of the 1,4-Dihydropyridine Moiety Where the Nitric Oxide Donor is Attached

In the case of the 1,4-dihydropyridine-organic nitrate (**6-16**) and 1,4-dihydropyridine-(*N*-nitrosoamine) hybrids (**46-48**) described in this thesis, the nitric oxide donor moiety can be attached either to the acetoacetate or aminocrotonate. The synthesis of an alkyl 3-aminocrotonate requires ammonia (Section 4.2.1.2.2). It was decided that the nitric oxide donor moiety should be coupled to the acetoacetate to avoid the possibility of a reaction with ammonia.

The preparation of 1,4-dihydropyridine-furoxan hybrids (**24-38**) is optimal when a (hetero)aryl adehyde (**4a-e**), 2-cyanoethyl 3-aminocrotonate (**21**) and nitroacetone (**20**) were condensed using the Two-Step Hantzsch Condensation method (Section 4.2.1.2). In contrast, when the condensation of a (hetero)aryl adehyde (**4a-e**), 2-cyanoethyl acetoacetate and 1-nitro-2-aminopropane was attempted, the reaction did not proceed. Through other divergent reactions, we observed that 1-nitro-2-aminopropane is not as reactive as nitroacetone (**20**). Most importantly, we noticed that 2-cyanoethyl 3-aminocrotonate (**21**) requires 20 hours to react instead of the typical 30 minutes. Thus, we concluded that 2-cyanoethyl acetoacetate is not very reactive. For this same reason, in the case of the N^1-substituted diazen-1-ium-1,2,-diolate hybrids (**57-65**), it was decided to use 2-cyanoethyl 3-aminocrotonate (**21**) rather than 2-cyanoethyl acetoacetate.

4.2.1.5 Synthesis and Separation of Chiral 1,4-Dihydropyridines

Needless to say, 1,4-dihydropyridines enantiomers are difficult to separate. Various have been proposed [Goldmann & Stoltefuss, 1991; Reeve *et al.*, 1992].

1. In the cases of 1,4-dihydropyridine carboxylic acids, a chiral base is added to the racemate of 1,4-dihydropyridine carboxylic acids. The diastereomeric salts are then separated by fractional recrystallization. Examples of chiral bases include cinchonidine, quinidine, cinchonine and brucine.
2. A secondary chiral centre is introduced in the 1,4-dihydropyridine commonly via either the C-3 or C-5 ester, or the N-1 amine. These 1,4-dihydropyridine diastereomers are then separated and the secondary chiral centre is removed.
3. A pig, rabbit or horse liver esterases used to selectively cleave the ester moiety of one enantiomer.
4. Separation using a chiral column.

Although, our research groups have synthesized chiral 1,4-dihydropyridine by the second method [Vo *et al.*, 1995], chiral synthesis and separation are not a part of this research project.

4.2.2 Synthesis of the Nitric Oxide Donor Moieties

This section (4.2.2) of the thesis will discuss the synthesis of the nitric oxide donor moieties.

4.2.2.1 Organic Nitrates

$$ROH + HNO_3 \xrightarrow{H_2SO_4} RONO_2 + H_2O$$

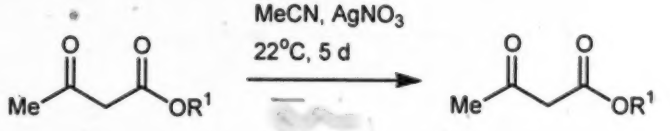
$$RX + AgNO_3 \longrightarrow RONO_2 + AgX \quad (X = I, Br, Cl)$$

Figure 4.2.2.1a: Two Methods to Synthesize Organic Nitrates

Nitrate preparation involves the esterification of an alkyl alcohol or the reaction of an alkyl halide with silver nitrate [Wang *et al.*, 2002] (Figure 4.2.2.1a).

Because the reactant for the esterification method is an alcohol, diacetoacetate by-products can be formed during the preparation of the nitrooxyalkyl acetoacetate (Section 4.2.1.2.1). Hence, for our organic nitrate hybrids (**6-16**), the substitution method was preferred.

Following an S_N2 mechanism [Carey & Sundberg, 1990], the method is time consuming requiring up to 5 days at 22°C in acetonitrile (Figure 4.2.2.1b). The S_N2 reaction did not occur for the conversion of 4-chlorobutyl acetoacetate (**2e**) to 4-nitrooxybutyl acetoacetate, and 3-isopropyl 5-(4-chlorobutyl) 1,4-dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**17**) was unintentionally prepared in the Hantzsch condensation reaction.



2a, $R^1 = (CH_2)_2Br$
2b, $R^1 = (CH_2)_3Br$
2c, $R^1 = (CH_2)_4Br$
2d, $R^1 = CH(CH_2Br)_2$
2e, $R^1 = (CH_2)_4Cl$

3a, $R^1 = (CH_2)_2ONO_2$
3b, $R^1 = (CH_2)_3ONO_2$
3c, $R^1 = (CH_2)_4ONO_2$
3d, $R^1 = CH(CH_2ONO_2)_2$

Figure 4.2.2.1b: Synthesis of Nitrooxyalkyl Acetoacetates (**3a-d**)

4.2.2.2 Furoxans

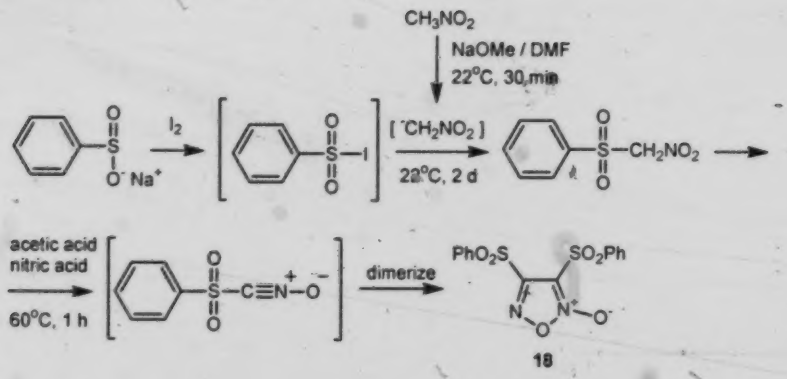


Figure 4.2.2.2: Synthesis of Bis(benzenesulfonyl)furoxan

There are several methods to prepare furoxans. Initial attempts to prepare (benzenesulfonyl)nitromethane using literature procedures [Kelley *et al.*, 1977; Wade *et al.*, 1981] were unsuccessful, until sodium hydride was used in place of sodium methoxide (Figure 4.2.2.2). (Benzenesulfonyl)nitromethane is a precursor to benezenesulfonylnitrile oxide that is produced upon heating in an acidic environment [Wang *et al.*, 2002]. The nitrile oxide dimerizes to form the desired bis(benzenesulfonyl)furoxan (**18**) which was subsequently used to synthesize our target 1,4-dihydropyridine-furoxan hybrids (**24-38**).

4.2.2.3 *N*-Nitrosoamines and N^1-Substituted Diazen-1-ium-1,2-diolates

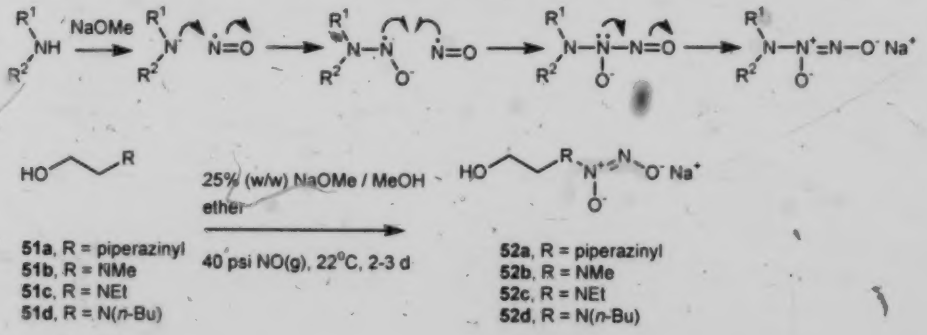


Figure 4.2.2.3a: Synthesis of O^2-sodium N^1-substituted diazen-1-ium-1,2-diolates (**52a-d**)

When a secondary amine (**51a-d**) is stirred under a nitric oxide atmosphere, an O^2-sodium N^1-substituted diazen-1-ium-1,2-diolate (**52a-d**) is formed, which precipitates from diethyl ether (Figure 4.2.2.3a).

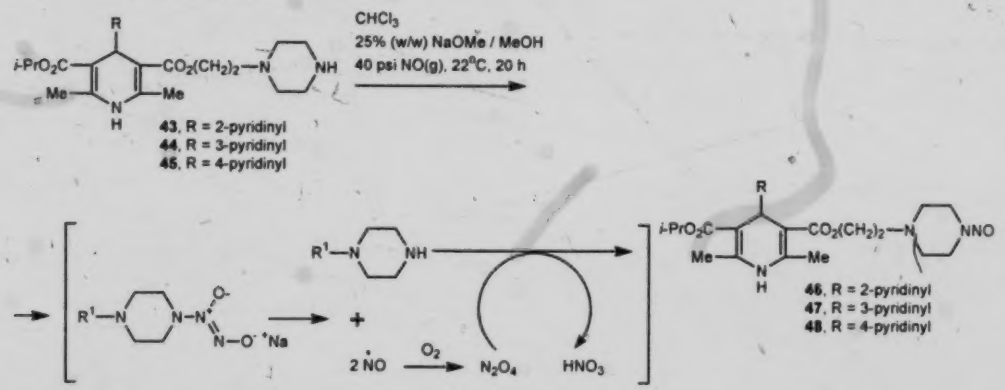


Figure 4.2.2.3b: Decomposition of 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 5-(2-[4-(O^2-Sodium Diazen-1-ium-1,2-diolate)piperazin-1-yl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates

In my studies, *N*-nitrosoamine products (**46-48**) were isolated due to the fact that the desired O^2-sodium N^1-substituted diazen-1-ium-1,2-diolate intially formed subsequently decomposed *in situ* to afford the *N*-nitrosoamine products (**46-48**) [Keefer, 1998] (Figure 4.2.2.3b). *N*-nitrosoamines are potential nitric oxide donors [Wang *et al.*, 2004].

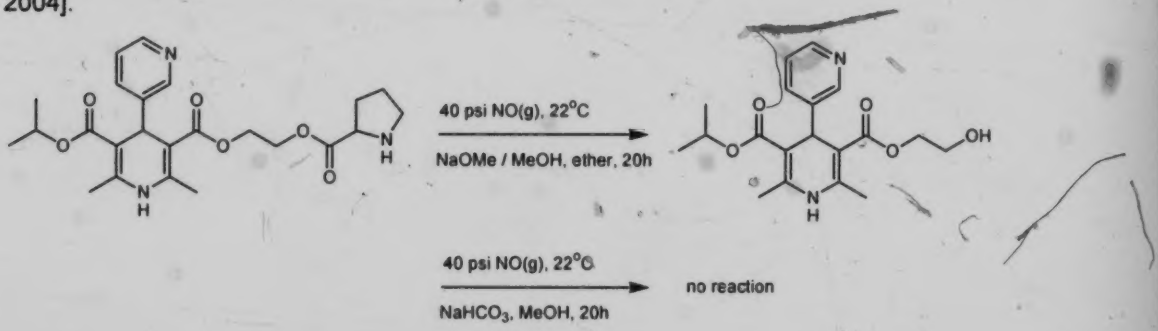


Figure 4.2.2.3c: Failed Synthesis of a 1,4-Dihydropyridine Possessing an Unprotected Pyrrolidinyl Moiety.

The synthesis of O^2-sodium (pyrrolidin-1-yl)diazen-1-ium-1,2-diolates or *N*-nitrosopyrrolidinyl derivatives were also attempted but these reactions did not afford the desired products (Figure 4.2.2.3c).

O^2-Sodium N^1-substituted diazen-1-ium-1,2-diolates are unstable under acidic conditions [Hrabie & Keefer, 2002]. It was noted that under prolonged exposure to air, the conjugate acids of compounds (**52a-d**) were formed, which subsequently decomposed.

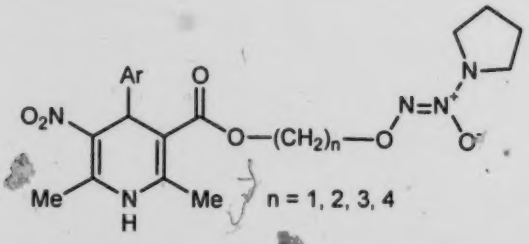


Figure 4.2.2.3d: 5-([(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate] O^2-alkyl) 1,4,-dihydro-2,6-dimethyl-3-nitro-4-(heteroaryl)-5-pyridinecarboxylates

O^2-Protected N^1-substituted diazen-1-ium-1,2-diolates were prepared to prevent decomposition. One of the members of our research group, Carlos Velázquez, synthesized the calcium channel agonists 5-([(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate] O^2-alkyl) 1,4,-dihydro-2,6-dimethyl-3-nitro-4-(heteroaryl)-5-pyridinecarboxylates [Velázquez *et al.*, 2003] (Figure 4.2.2.3d). Studies were initiated using the dicarboxylate calcium channel antagonist derivatives of the compounds Velázquez had prepared, but were promptly terminated when it was learned that this specific type of diazen-1-ium-1,2-diolate did not release nitric oxide.

To prevent the immediate release of nitric oxide, an O^2-sodium salt of 1-(*N*-substituted)diazen-1-ium-1,2-diolate can be converted to a biolabile O^2-acetoxymethyl 1-(*N*-substituted)diazen-1-ium-1,2-diolate derivative that is stable in neutral aqueous media, but which released nitric oxide upon metabolism (ester cleavage) by esterases [Saavedra *et al.*, 2000].

An attempt to synthesize O^2-acetoxymethyl (2-hydroxymethylpyrrolidin-1-yl)diazen-1-ium-1,2,-diolate was unsuccessful (Figure 4.2.2.3d). Consequently, a tosyl protective group was investigated. Unfortunately, it was not possible to couple this

moiety to the 1,4-dihydropyridine. At the time, we had not yet developed reaction conditions for this coupling reaction. Coupling will be discussed further in Section 4.2.3.

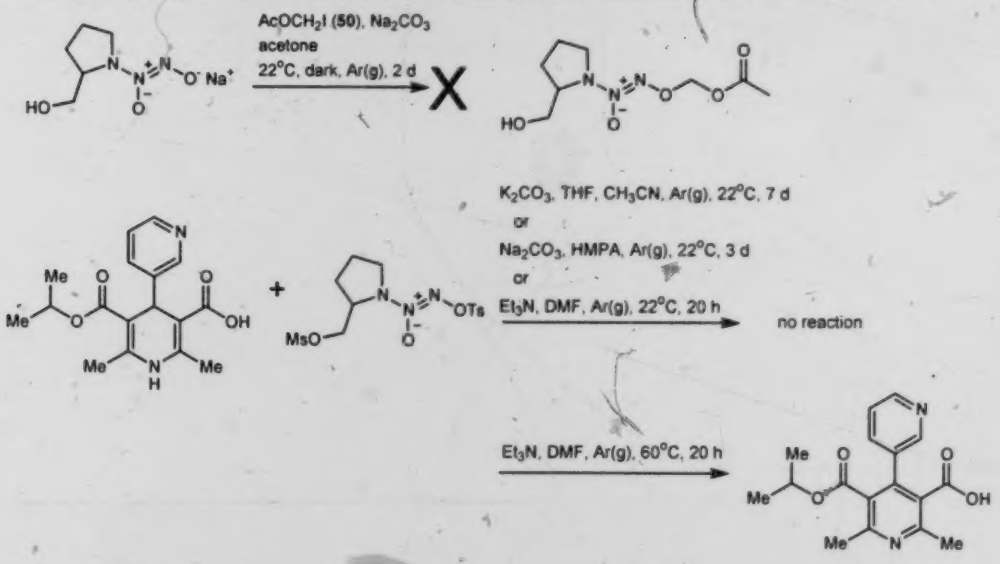


Figure 4.2.2.3d: Failed Synthesis of a 1,4-Dihydropyridine Possessing a Protected Pyrrolidinyl Moiety

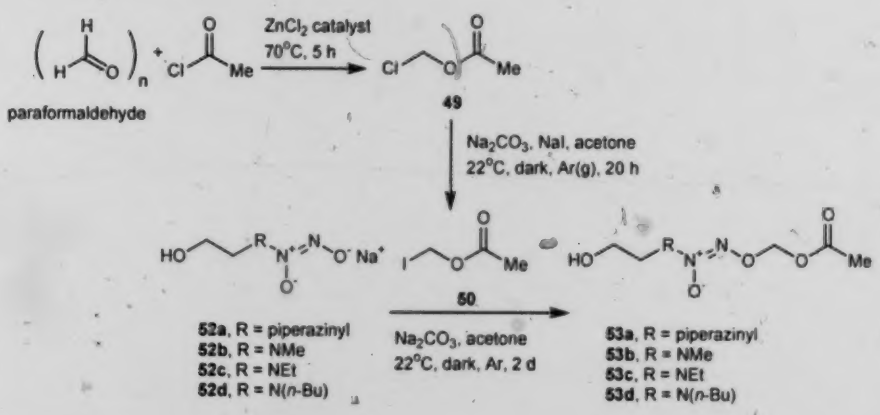


Figure 4.2.2.3e: Synthesis of O^2-Acetoxymethyl N^1-Substituted Diazen-1-ium-1,2-diolates (**53a-d**)

Eventually, it was decided to use other secondary amines to synthesize compounds **53a-d** (Figure 4.2.2.3e). At first, compounds **53c-d** were prepared

according to reported procedures [Velázquez & Knaus, 2004], but were obtained in fairly low yields (**53c**, 9%; **53d**, 8%). It was believed that the presence of the by-product hydroiodic acid decomposed the diazen-1-ium-1,2-diolate, since nitric oxide release from diazen-1-ium-1,2-diolate is reported to be enhanced under acidic condition [Hrabie & Keefer, 2002]. Consequently, excess sodium carbonate was added to maintain a basic medium, and the yields were higher (**53c**, 30%; **53d**, 40%). O^2-Sodium 1-(*N*-[2-hydroxyethyl]-*N*-(*tert*-butylamino)diazen-1-ium-1,2,-diolate [R = N(*t*-Bu)] was prepared but its conversion to the O^2-acetoxymethyl protected derivative was unsuccessful.

4.2.3 Coupling the Nitric Oxide Donor to the 1,4-Dihydropyridine

Section 4.2.1 described the synthesis of the 1,4-dihydropyridine moiety. Section 4.2.2 explained the preparation of the nitric oxide donor moiety. The complete synthesis of organic nitrate (**6-16**) and *N*-nitrosoamine hybrids (**46-48**) have been described. This section (4.2.3) of the thesis will discuss the attachment of the nitric oxide donor moiety to the 1,4-dihydropyridine moiety, and is of relevance to the furoxan (**24-38**) and N^1-substituted diazen-1-ium-1,2-diolate (**57-65**) hybrids.

During the preparation of the furoxan (**24-38**) and N^1-substituted diazen-1-ium-1,2-diolate (**57-65**) hybrids, the nitric oxide donor and the 1,4-dihydropyridine moieties were prepared independently. With this technique, failed reaction steps were more easily "back-tracked".

4.2.3.1 Conversions of 5-(2-Cyanoethyl) 1,4-Dihydropyridine-5-carboxylates to 1,4-Dihydropyridine-5-carboxylic Acids

5-(2-Cyanoethyl) 1,4-dihydropyridine-5-carboxylate compounds (**22a-e** and **55a-c**) were converted to 1,4-dihydropyridine-5-carboxylic acids (**23a-e** and **56a-c**) (Figure 4.2.3.1a). As discussed in 1.4.1.6, hydrolysis of the ester is not easily achieved. In the cases of compounds **22a-e**, we used DBU to remove the cyanoethyl moiety via a β-elimination reaction mechanism [Goldmann & Stoltefuss, 1991]. Alternatively, in the cases of compounds **55a-c**, it was found that prolonged exposure to a large access of sodium methoxide also cleaved the cyanoethyl ester, providing higher product yields. In contrast, an attempt to hydrolyze the methyl ester of BAY K 8644 with sodium methoxide was not successful which suggests the sodium methoxide mechanism for cleaving the cyanoethyl ester is β-elimination rather than hydrolysis.

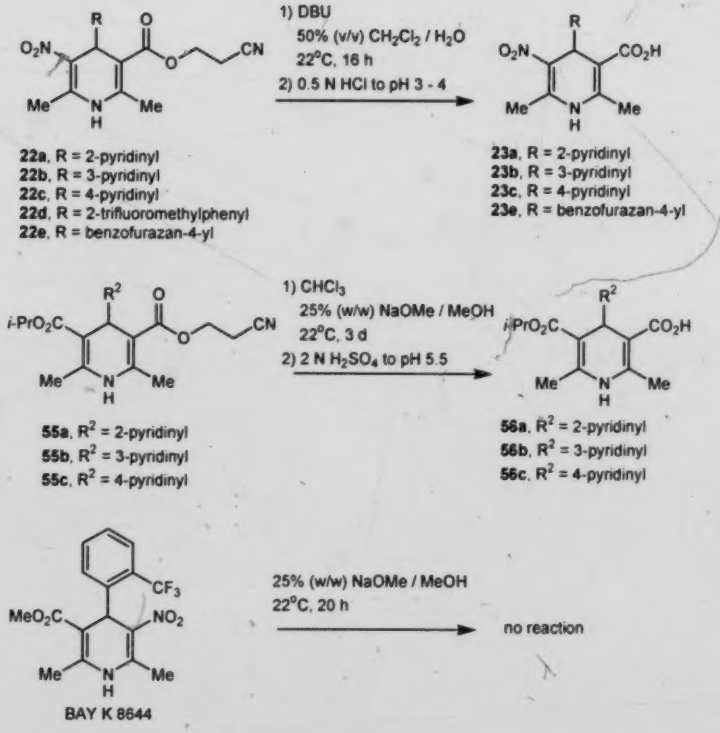

1) DBU
50% (v/v) CH2Cl2 / H2O
22°C, 16 h
2) 0.5 N HCl to pH 3 - 4
22a, R = 2-pyridinyl
22b, R = 3-pyridinyl
22c, R = 4-pyridinyl
22d, R = 2-trifluoromethylphenyl
22e, R = benzofurazan-4-yl
23a, R = 2-pyridinyl
23b, R = 3-pyridinyl
23c, R = 4-pyridinyl
23e, R = benzofurazan-4-yl
1) CHCl3
25% (w/w) NaOMe / MeOH
22°C, 3 d
2) 2 N H2SO4 to pH 5.5
55a, R2 = 2-pyridinyl
55b, R2 = 3-pyridinyl
55c, R2 = 4-pyridinyl
56a, R2 = 2-pyridinyl
56b, R2 = 3-pyridinyl
56c, R2 = 4-pyridinyl
25% (w/w) NaOMe / MeOH
22°C, 20 h
no reaction
BAY K 8644

Figure 4.2.3.1a: Synthesis of 1,4-Dihydropyridine-5-carboxylic Acids (**23a-e** and **56a-c**)

It was also observed that when the carboxylic acid is exposed to an organic base such as triethylamine in DMF at 60°C for 20 hours, or pyridine at 22°C for 1 – 6 days, that the 1,4-dihydropyridine ring was aromatized (Figure 4.2.3.1b).

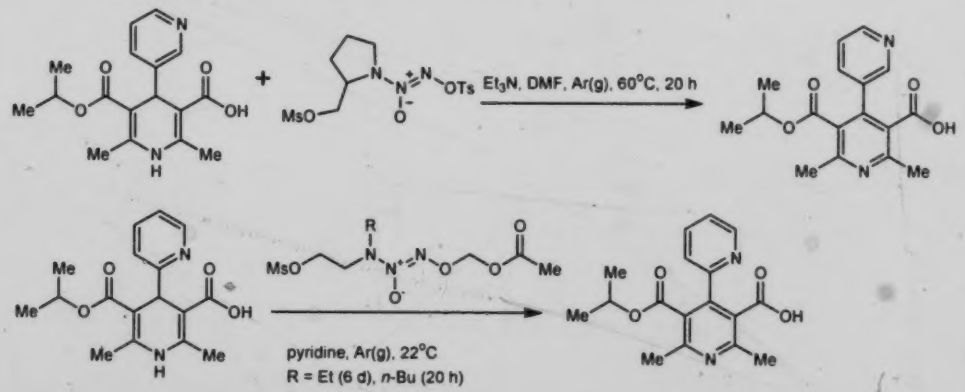
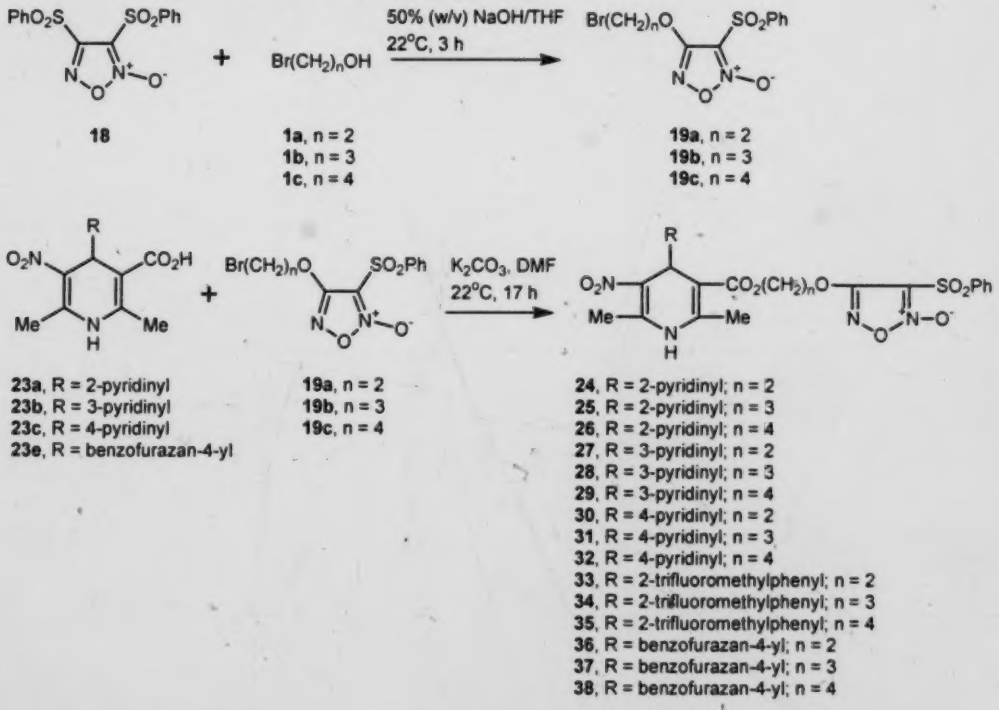


Figure 4.2.3.1b: Aromatizations of 1,4-Dihydropyridine-3-isopropyloxycarbonyl-4-(pyridinyl)-5-carboxylic Acids

4.2.3.2 Synthesis of 1,4-Dihydropyridine-Furoxan Hybrids



18

1a, n = 2
1b, n = 3
1c, n = 4

19a, n = 2
19b, n = 3
19c, n = 4

23a, R = 2-pyridinyl
23b, R = 3-pyridinyl
23c, R = 4-pyridinyl
23e, R = benzofurazan-4-yl

19a, n = 2
19b, n = 3
19c, n = 4

24, R = 2-pyridinyl; n = 2
25, R = 2-pyridinyl; n = 3
26, R = 2-pyridinyl; n = 4
27, R = 3-pyridinyl; n = 2
28, R = 3-pyridinyl; n = 3
29, R = 3-pyridinyl; n = 4
30, R = 4-pyridinyl; n = 2
31, R = 4-pyridinyl; n = 3
32, R = 4-pyridinyl; n = 4
33, R = 2-trifluoromethylphenyl; n = 2
34, R = 2-trifluoromethylphenyl; n = 3
35, R = 2-trifluoromethylphenyl; n = 4
36, R = benzofurazan-4-yl; n = 2
37, R = benzofurazan-4-yl; n = 3
38, R = benzofurazan-4-yl; n = 4

Figure 4.2.3.2: Coupling Reactions of 3-Benzenesulfonyl-4-(bromoalkoxy)furoxans (**19a-c**) to 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(aryl)-5-pyridinecarboxylic Acids (**23a-e**)

Bis(benzenesulfonyl)furoxan (**18**) was converted to 3-benzenesulfonyl-4-(bromoalkoxy)furoxans (**19a-c**). The reaction conditions were critical, if one desired an asymmetrical product rather than a symmetrical product [Sorba *et al.*, 1996].

Coupling of the 1,4-dihydropyridine-5-carboxylic acid (**23a-e**) with the bromoalkyl compound (**19a-c**) occured via a substitution mechanism.

4.2.3.3 Synthesis of 1,4-Dihydropyridine-(N^1-Substituted Diazen-1-ium-1,2-diolate) Hybrids

Attempts to activate the 1,4-dihydropyridine-5-carboxylic acid such that it undergoes nucleophilic acyl substitution with the hydroxyethyl group of the nitric oxide donor were unsuccessful (Figure 4.2.3.3a). Coupling the acid chloride, prepared by

reaction with oxalyl chloride, did not afford the expected product. An attempt to couple the acid (**56a**) to the alcohol (**53d**) using cyclohexylcarbodiimide (CCD) was also unsuccessful.

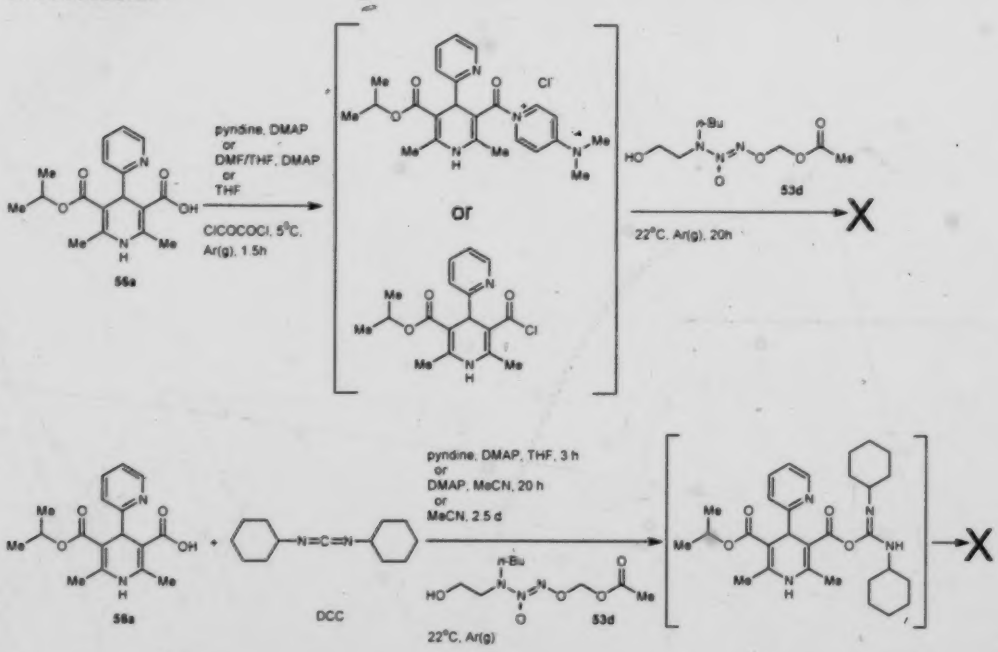


Figure 4.2.3.3a: Failed Activations of 1,4-Dihydropyridine-3-isopropyloxycarbonyl-4-(2-pyridinyl)-5-carboxylic Acid

An alternative strategy uses the 1,4-dihydropyridine-5-carboxylic acid (**56a-c**) as a nucleophile rather than an electrophile (Figure 4.2.3.3b). Mesylation of the hydroxyalkyl group of the nitric oxide donor (**54a-d**) and its prompt coupling to the 1,4-dihydropyridine-5-carboxylic acid (**56a-c**) was investigated. When the coupling reaction was carried out at 22°C in the presence of sodium carbonate in HMPA, DMF, THF, acetonitrile or acetone for times up to 7 days, no reaction occurred. When the reaction was performed using an organic base such as pyridine or triethylamine, the desired product was not obtained, due to oxidation of the 1,4-dihydropyridine ring to the corresponding aromatic pyridine product (Figure 4.2.3.1b). Fortunately, when the reaction with sodium carbonate was performed in acetonitrile at 50°C, the desired products (**58-65**) were obtained in optimal yields after 5 days. However, the piperazinyl compound (**57**) could not be prepared using similar conditions. Compound **57** was

obtained when the reaction was done in acetonitrile at 70°C for 2 days. If the reaction continued for more than 2 days, the desired product (**57**) would decompose. The stability of N^1-substituted diazen-1-ium-1,2-diolate hybrids (**57-65**) will be elaborated in Section 4.2.4.3.

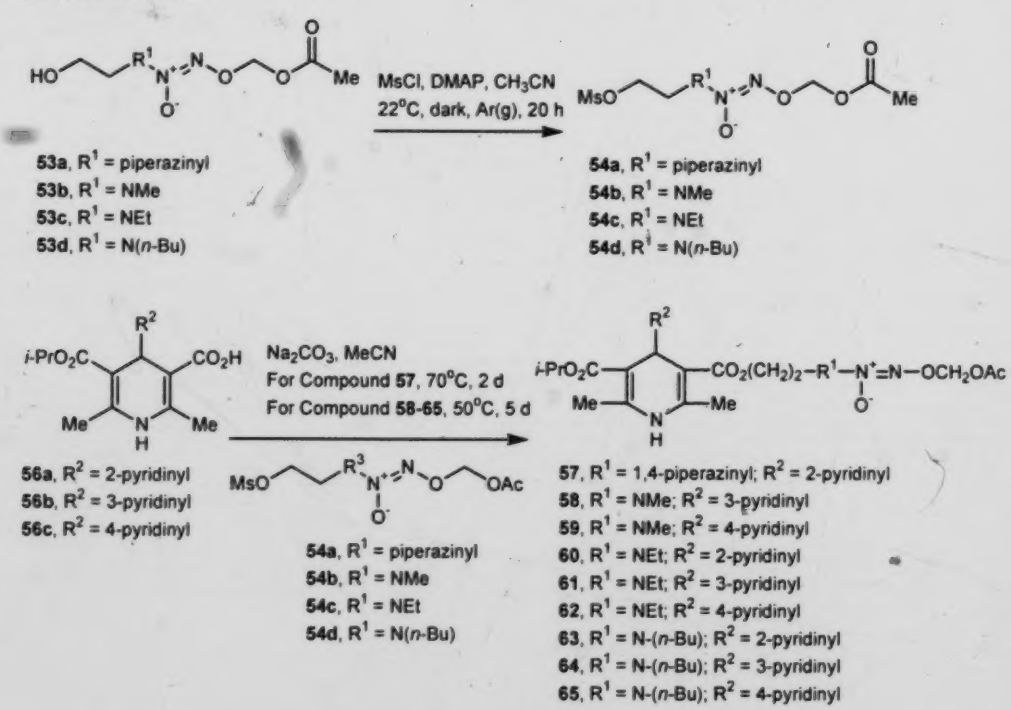

MsCl, DMAP, CH3CN
22°C, dark, Ar(g), 20 h
53a, R1 = piperazinyl
53b, R1 = NMe
53c, R1 = NEt
53d, R1 = N(n-Bu)
54a, R1 = piperazinyl
54b, R1 = NMe
54c, R1 = NEt
54d, R1 = N(n-Bu)
Na2CO3, MeCN
For Compound 57, 70°C, 2 d
For Compound 58-65, 50°C, 5 d
56a, R2 = 2-pyridinyl
56b, R2 = 3-pyridinyl
56c, R2 = 4-pyridinyl
54a, R1 = piperazinyl
54b, R1 = NMe
54c, R1 = NEt
54d, R1 = N(n-Bu)
57, R1 = 1,4-piperazinyl; R2 = 2-pyridinyl
58, R1 = NMe; R2 = 3-pyridinyl
59, R1 = NMe; R2 = 4-pyridinyl
60, R1 = NEt; R2 = 2-pyridinyl
61, R1 = NEt; R2 = 3-pyridinyl
62, R1 = NEt; R2 = 4-pyridinyl
63, R1 = N-(n-Bu); R2 = 2-pyridinyl
64, R1 = N-(n-Bu); R2 = 3-pyridinyl
65, R1 = N-(n-Bu); R2 = 4-pyridinyl

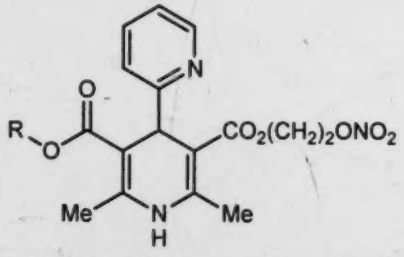
Figure 4.2.3.3b: Coupling Reactions of O^2-acetoxymethyl 1-([2-methylsulfonyloxyethyl][*N*,*N*-dialkylamino or 4-piperazin-1-yl])diazen-1-ium-1,2,-diolate (**54a-d**) to 1,4-Dihydro-2,6-dimethyl-3-isopropyloxycarbonyl-4-(pyridinyl)-5-pyridinecarboxylic Acids (**56a-c**)

4.2.4 Physicochemical and Structural Features of the 1,4-Dihydropyridine Calcium Channel Modulators Synthesized

This section (4.2.4) of the thesis will discuss various physicochemical and structural features of the 1,4-dihydropyridine calcium channel modulators synthesized (**6-17**, **24-38**, **43-44**, **46-48**, and **57-65**).

4.2.4.1 1,4-Dihydropyridine-Organic Nitrate Hybrids Investigated

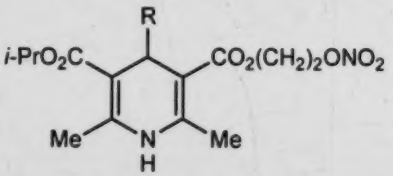
The preparations of the organic nitrate hybrids (**6-16**) were done simultaneously with the pharmacological tests. Consequently, the specific 1,4-dihydropyridines prepared were dictated by the pharmacological results. Pharmacological results will be discussed in details in Section 4.3.



R = Et (**6**), *i*-Pr (**7**), *i*-Bu (**10**), *t*-Bu (**11**)

Figure 4.2.4.1a: 3-Nitrooxyethyl 5-Alkyl 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylates (**6-7** and **10-11**)

First, we investigated the effect of an alkyl substitution on the "essential side" of the calcium channel antagonist, and observed that isopropyl substitution greatly contributed to antagonist activity (*i*-Pr >> Et > *t*-Bu > *i*-Bu) (Figure 4.2.4.1a).



R = 2-, 3-, 4-pyridinyl (**7-9**)

Figure 4.2.4.1b: 3-Isopropyl 5-Nitrooxyethyl 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**7-9**)

Hence, we retained the isopropyl substituent and investigated the effect of pyridinyl substitution at the C-4 position (Figure 4.2.4.1b). Pyridinyl substitution followed the general pattern of 2-pyridinyl > 3-pyridinyl > 4-pyridinyl in regards to calcium channel antagonist potency.

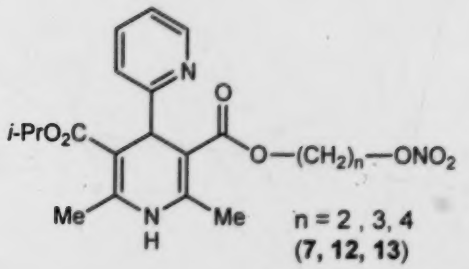


Figure 4.2.4.1c: 3-Isopropyl 5-Nitrooxyalkyl 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylates (**7, 12, 13**)

Next, the 2-pyridinyl substituent was maintained along with the isopropyl substituent, and the chain attached to the nitrooxy group was elongated (Figure 4.2.4.1c). The effect of chain elongation on the nitrooxy substituent followed the general pattern of n = 2 > 3 > 4 in terms of calcium channel antagonist activity. The observed general pattern does not agree with Gasco *et al.* (1996)'s report that the *in vitro* vasodilating activity of aliphatic nitrates is linearly dependent on lipophilicity. We did not quantify for lipophilicity in our group of compounds.

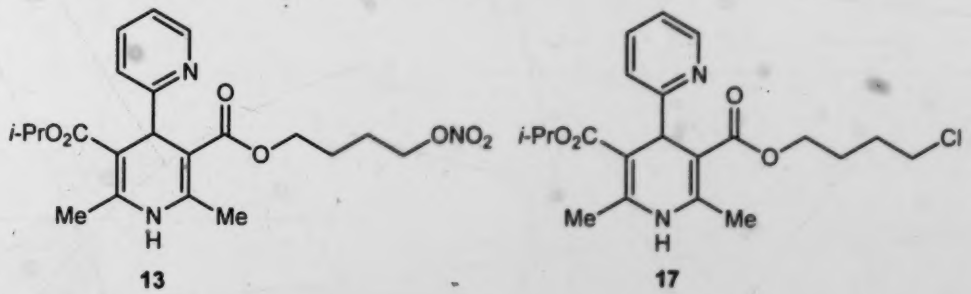


Figure 4.2.4.1d: 3-Isopropyl 5-Nitrooxybutyl 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**13**) and 3-Isopropyl 5-Chlorobutyl 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**17**)

To ascertain whether the nitric oxide released from the compounds had an effect on calcium channel antagonism, we synthesized a chloro substituted compound (**17**) to compare with a similar nitrooxy substituted compound (**13**) (Figure 4.2.4.1d). The nitric oxide released from compound **13** (0.04%) did not seem to affect calcium channel antagonism.

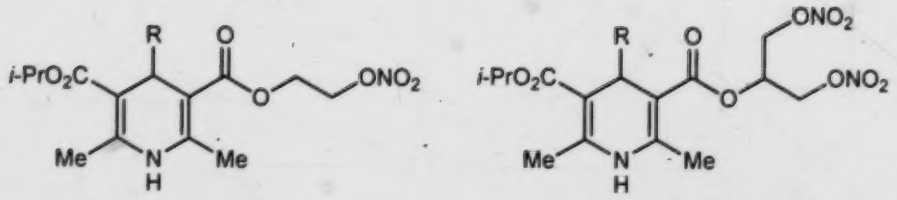
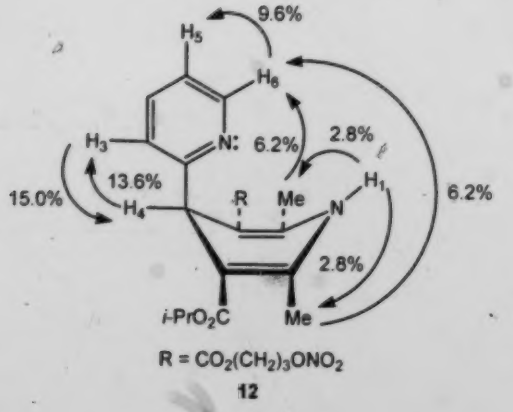


Figure 4.2.4.1e: 3-Isopropyl 5-Nitrooxyalkyl 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**7-9** and **14-16**)

The 1,4-dihydropyridines with a single nitrooxy group attached released very little nitric oxide (0.034% to 0.096% per mol; glyceryl trinitrate, 11.427%). To mimic glyceryl trinitrate, in which nitric oxide release is dependent on steric interactions between adjacent nitrooxy groups (Section 1.5.2), 1,4-dihydropyridines with two adjacent nitrooxy moieties were synthesized (Figure 4.2.4.1e). The di-nitrooxy 1,4-dihydropyridines (**14-16**) released a higher percentage of nitric oxide (0.580% to 0.836%) than the mono-nitrooxy 1,4-dihydropyridines (**7-9**) (0.034% to 0.096%) but released considerably lower than glyceryl trinitrate (11.427%). Overall, the di-nitrooxy 1,4-dihydropyridines (**14-16**) were less potent calcium channel antagonists than the mono-nitrooxy 1,4-dihydropyridines (**7-9**).



12

Figure 4.2.4.1f: Some Nuclear Overhauser Enhancement Determinations to Study the Rotameric Orientation of the 2-Pyridinyl Moiety for 3-Isopropyl 5-(3-Nitrooxypropyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**12**) in $CDCl_3$ at 22°C.

A low-temperature ^{1}H NMR spectrum of 3-isopropyl 5-(3-nitrooxypropyl) 1,4-dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**12**) at -30°C in $CDCl_3$ did not give rise to any dual resonances. Although this absence of dual resonances indicates the possibility that a single rotamer and/or preference for the C-4 2-pyridinyl moiety exists in solution, it is equally plausible that the rotation barrier is too small to stop rotation at -30°C, or that there is a thermodynamic preference for one rotamer regardless of the magnitude of the energy barrier to rotation. However, it has been reported [Goldmann & Geiger, 1984] that the two o-methyl resonances for dimethyl 1,4-dihydro-2,6-dimethyl-4-(2,4,6-trimethylphenyl)-3,5-pyridinedicarboxylate appear as a broad singlet in $CDCl_3$ at 25°C, coalescence occurred at -18°C (ΔG(-18°C) = 51.1 kJ/mol), and two separate resonances were observed at -50°C. It was therefore of interest to determine the rotameric orientation of the 2-pyridinyl ring system present in compound **12** in solution ($CDCl_3$ at 22°C) (Figure 4.2.4.1f). The results from ^{1}H NMR nuclear Overhauser enhancement (nOe) studies clearly indicate that a predominant rotamer exists in solution where the pyridinyl nitrogen atom is orientated above the 1,4-dihydropyridine ring system (6.2% nOe enhancement from either the C-2, or C-6, methyl group to the pyridinyl H-6), and that the pyridinyl nitrogen atom is *anti* to the 1,4-dihydropyridine H-4 (15.0% nOe enhancement from the pyridinyl H-3 to the 1,4-dihydropyridine H-4), and the nitrogen free-electron pair is orientated above the plane of the 1,4-dihydropyridine ring system. In simpler words, the 2-pyridinyl ring is in an *anti* rotameric orientation (Section 1.4.1.2), thus agreeing with earlier nOe [Iqbal *et al.*, 1998] and X-ray crystallographic [Parrish *et al.*, 2000] studies.

Semi-empirical molecular orbital (AM1, PM3) conformational analyses of 1,4-dihydropyridine modulators provide an attractive method to model 1,4-dihydropyridine geometry [Bikker & Weaver, 1993]. The PM3-geometry optimized structure for compound **12** revealed the interatomic distance between the amine and pyridinyl nitrogen atoms (3.39 Å) is within hydrogen-bonding range, and that this interatomic distance is less than that between the *N*H nitrogen and the closest carbonyl oxygen atom (4.36 Å) (Figure 4.2.4.1g). An intramolecular hydrogen-bond between the 2-pyridinyl nitrogen free electron-pair and the N*H* would maintain the 2-pyridinyl nitrogen atom in the observed *anti* rotameric orientation to the 1,4-dihydropyridine H-4 proton [Iqbal *et al.*, 1998]. However, this explanation is unlikely since the X-ray crystal structure of isopropyl 1,4-dihydro-2,6-dimethyl-4-(6-methyl-2-pyridinyl)-3-nitro-5-

pyridinecarboxylate showed the absence of an intramolecular hydrogen-bond between the 1,4-dihydropyridine N*H* and the pyridinyl nitrogen atom, since the N-H bond vector is orientated in the same direction as the pyridinyl nitrogen free electron-pair [Parrish *et al.*, 2000]. In contrast, this crystal structure showed intermolecular hydrogen-bonds were present between the two nitrogen-atoms (pyridinyl nitrogen and 1,4-dihydropyridine N*H*) indicating intermolecular packing interactions in the X-ray crystal structure. The possibility of an intermolcular hydrogen-bonded dimer persisting in solution similar to that observed in the solid state is supported by the ^{1}H NMR hydrogen-bonding data described above. Accordingly, the observed *anti* rotameric orientation of the pyridinyl nitrogen-atom in compound **12** could be due to the smaller relative size of the pyridinyl ring nitrogen-atom relative to the pyridinyl ring C-3 carbon- and hydrogen-atoms, or alternatively there may be an electrostatic attraction between the pyridinyl nitrogen free electron-pair and the 1,4-dihydropyridine moiety.

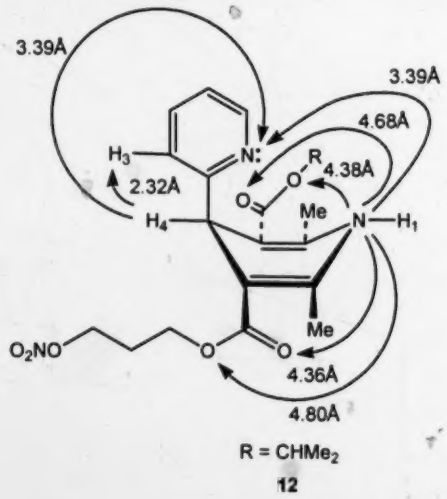


Figure 4.2.4.1g: Some Interatomic Distances for the PM3-Geometry Optimized Structure of 3-Isopropyl 5-(3-Nitrooxypropyl) 1,4-Dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**12**)

Variable temperature ^{1}H NMR spectroscopy is a useful method to study hydrogen-bonding interactions. Lowering the temperature may stop N*H* exchange (gives rise to a sharp, or coupled, NH resonance), which can enhance hydrogen-bonding that results in a deshielding (down-field shift) for the N*H* proton. Conversely, increasing temperature may disrupt hydrogen-bonding resulting in a more rapid rate of N*H*

exchange (gives rise to a broader resonance) that results in an upfield shift (shielding effect) for the N*H* proton due to disruption of hydrogen-bonding [Jackman & Sternhell, 1969]. It was observed (^{1}H NMR spectra) that the chemical shift for the N*H* proton of compound **12** in $CDCl_3$ was highly temperature-dependent. For example, the N*H* proton for compound **12** appeared at δ 10.15 (-30°C), 9.41 (0°C), 8.46 (25°C), 8.15 (40°C), and 7.24 (60°C) (Figure 4.2.4.1h). All other resonances for compound **12** showed minor changes in chemical shift positions irrespective of temperature. These N*H* chemical shift dependence data indicate that the N*H* group must be hydrogen-bonded (sharper N*H*, more deshielded) at lower temperature, and that hydrogen-bonding is disrupted (broader N*H*, more shielded) upon heating to 60°C.

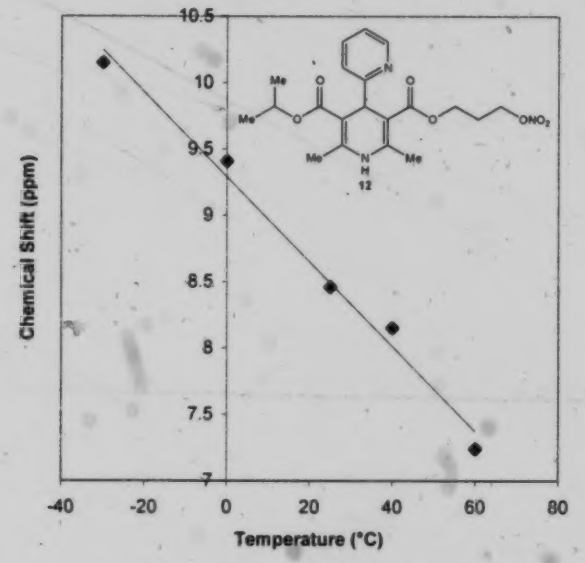


Figure 4.2.4.1h: A Graph Displaying the Change in ^{1}H NMR Chemical Shift of the N*H* Proton of Compound **12** as the Temperature is Increased.

4.2.4.2 1,4-Dihydropyridine-Furoxan Hybrids Investigated

There is significantly less literature describing 1,4-dihydropyridine calcium channel agonists than antagonists. A novel class of 1,4-dihydropyridine calcium channel agonist-furoxan hybrids was synthesized (**24-38**) (Figure 4.2.4.2a). In pursuit of nitric oxide donor-calcium channel agonist hybrids, a 4-benzofurazanyl or 2-trifluoromethylphenyl substituent was attached to the C-4 position based on the classical

1,4-dihydropyridine calcium channel agonists PN 202 791 and BAY K 8644, respectively, along with pyridinyl substituents.

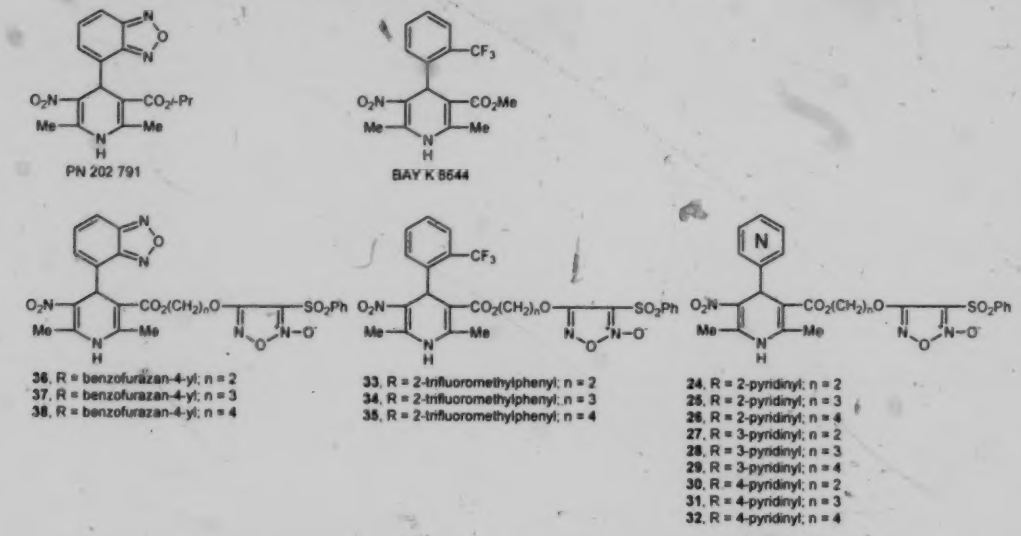

PN 202 791
BAY K 8644
36, R = benzofurazan-4-yl; n = 2
37, R = benzofurazan-4-yl; n = 3
38, R = benzofurazan-4-yl; n = 4
33, R = 2-trifluoromethylphenyl; n = 2
34, R = 2-trifluoromethylphenyl; n = 3
35, R = 2-trifluoromethylphenyl; n = 4
24, R = 2-pyridinyl; n = 2
25, R = 2-pyridinyl; n = 3
26, R = 2-pyridinyl; n = 4
27, R = 3-pyridinyl; n = 2
28, R = 3-pyridinyl; n = 3
29, R = 3-pyridinyl; n = 4
30, R = 4-pyridinyl; n = 2
31, R = 4-pyridinyl; n = 3
32, R = 4-pyridinyl; n = 4

Figure 4.2.4.2a: 5-([3-Benzenesulfonyl]furoxan-4-yloxy)alkyl 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl, benzofurazan-4-yl, 2-, 3- or 4-pyridinyl)-5-pyridinecarboxylates (**24-38**)

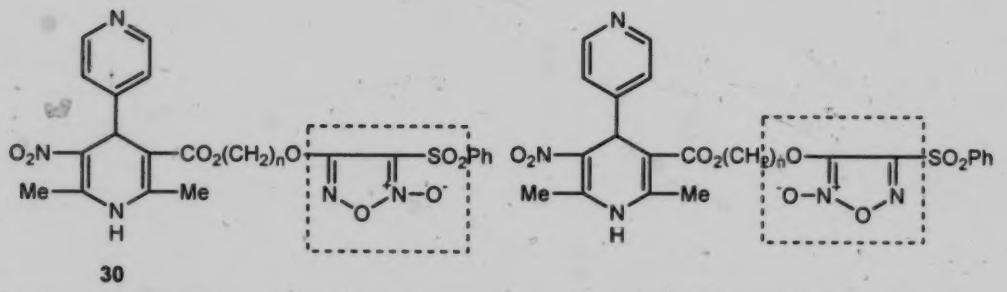


Figure 4.2.4.2b: 1,4-Dihydro-3-nitropyridine Calcium Channel Agonists Possessing Either a (3-[Benzenesulfonyl]furoxan-4-yloxy)alkyl or (4-[Benzenesulfonyl]furoxan-3-yloxy)alkyl Moiety

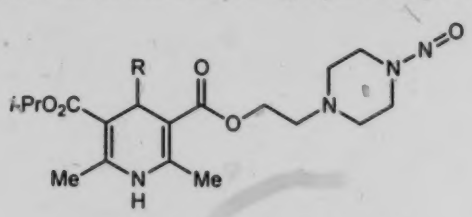
The ^{13}C NMR spectrum for compound **30** was acquired to determine whether the ester substituent present in compounds **24-38** existed as the [3-

(benzenesulfonyl)furoxan-4-yloxy]alkyl, or the [4-(benzenesulfonyl)furoxan-3-yloxy]alkyl isomer (Figure 4.2.4.2b). The appearance of the furoxan *C*-3 resonance at δ 110.84, and the furoxan *C*-4 resonance at δ 158.26, is consistent with ^{13}C NMR data reported for similar [3-(benzenesulfonyl)furoxan-4-yloxy]alkyl compounds [Fruttero *et al.*, 1997].

4.2.4.3 1,4-Dihydropyridine-(*N*-Nitrosoamine) and (*N*^{1}-Substituted Diazen-1-ium-1,2-diolate) Hybrids Investigated

In previous studies investigating organic nitrate hybrids (**6-16**), it was learned that an isopropyl ester substituent at the C-3 position greatly enhanced calcium channel antagonist activity (Section 4.2.4.1). Consequently, we maintained the C-3 isopropyl ester substituent in our attempts to synthesize 1,4-dihydropyridine-(*N*^{1}-substituted diazen-1-ium-1,2-diolate) hybrids (**57-65**).

4.2.4.3.1 1,4-Dihydropyridine-(*N*-Nitrosoamine) Hybrids Synthesized



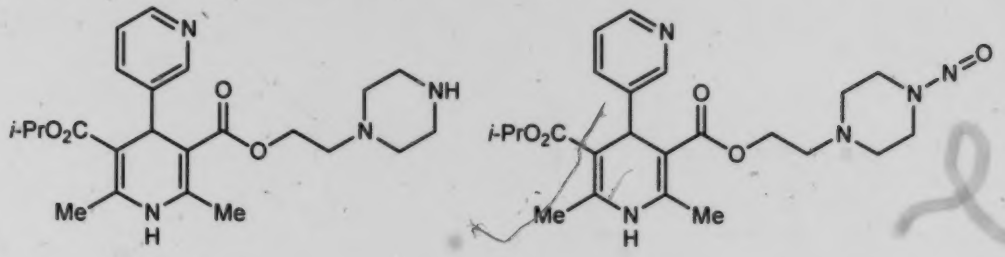
R = 2-, 3-, 4-pyridinyl (**46-48**)

Figure 4.2.4.3.1: 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**46-48**)

In an earlier discussion (Section 3.1.3), it was mentioned that one of the methods employed to synthesize *N*^{1}-substituted diazen-1-ium-1,2-diolate hybrids culminated in the formation of *N*-nitrosoamine 1,4-dihydropyridines (**46-48**) (Figure 4.2.4.3.1a). In addition to IR and ^1H NMR spectroscopy, the purity and identity of compounds **46-48** were verified by ^{13}C NMR and mass spectroscopy.

The differences between empirical and theoretical microanalytical values of compounds **46-48** were greater than 0.4%. The ^1H NMR spectra of compounds **46-48** suggested that the samples had only minute amount of proton-containing impurities. The fragmentation patterns, as determined by positive field electrospray mass

spectrometry, confirmed the identities of the compounds.



44 **47**

Figure 4.2.4.3.1b: 3-Isopropyl 5-(2-Piperazinylethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (**44**) and 3-Isopropyl 5-(2-[4-Nitrosopiperazinyl]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(3-pyridinyl)-3,5-pyridinedicarboxylate (**47**)

A comparison of the ^{13}C NMR spectrum of compound **47** with that of its unsubstituted piperazinyl precursor **44** revealed that the *N*-nitroso substituent in compound **47** on the piperazinyl ring confers chemical shift non-equivalence on the C-3 and C-5 piperazinyl resonances. A ^{1}H NMR decoupling study on compound **47** aided in assigning chemical shifts by highlighting neighboring protons, and permitted interpretation of the complicated coupling pattern of the piperazinyl protons.

Compounds **46-48** were subjected to pharmacological testing because *N*-nitrosoamines are potential nitric oxide donors [Wang *et al.*, 2002].

4.2.4.3.2 1,4-Dihydropyridine-(*N*^{1}-Substituted Diazen-1-ium-1,2-diolate) Hybrids Synthesized

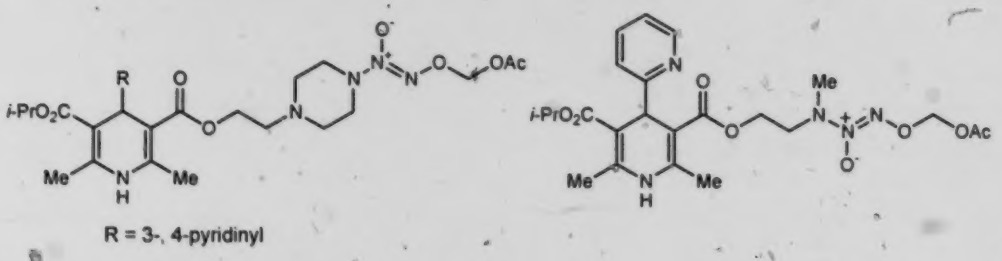
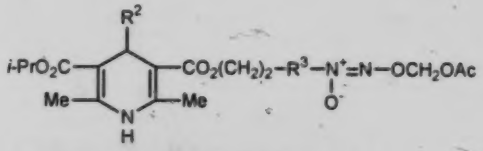


Figure 4.2.4.3.2a: Unstable 1,4-Dihydropyridine-(*N*^{1}-Substituted Diazen-1-ium-1,2-diolate) Hybrids

During the preparation and purification of N^1-substituted diazen-1-ium-1,2-diolate hybrids (**57-65**), it was observed that these hybrids were fairly unstable, especially the piperazinyl (**57**) and *N*-methyl hybrids (**58-59**). On numerous occasions, 5-(2-[4-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)piperazin-1-yl]ethyl) 1,4-dihydro-2,6-dimethyl-4-(3- or 4-pyridinyl)-3,5-pyridinedicarboxylates, and 5-(2-[*N*-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-methylamino]ethyl) 1,4-dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate were synthesized (Figure 4.2.4.3.2a). They decomposed during purification by silica gel column chromatography. Indeed, we also observed that 5-(2-[4-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)piperazin-1-yl]ethyl) 1,4-dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate decomposed when it was dried at 60°C for 30 minutes using benchtop vacuum (5 – 10 mmHg). The decompositions were determined by ^{1}H NMR.



57, R^2 = 2-pyridinyl; R^3 = 1,4-piperazinyl
58, R^2 = 3-pyridinyl; R^3 = NMe
59, R^2 = 4-pyridinyl; R^3 = NMe
60, R^2 = 2-pyridinyl; R^3 = NEt
61, R^2 = 3-pyridinyl; R^3 = NEt
62, R^2 = 4-pyridinyl; R^3 = NEt
63, R^2 = 2-pyridinyl; R^3 = N-(*n*-Bu)
64, R^2 = 3-pyridinyl; R^3 = N-(*n*-Bu)
65, R^2 = 4-pyridinyl; R^3 = N-(*n*-Bu)

Figure 4.2.4.3.2b: 3-Isopropyl 5-(2-[(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)(*N*,*N*-dialkylamino or 4-piperazin-1-yl)]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**57-65**)

During the course of this investigation, N^1-substituted diazen-1-ium-1,2-diolate hybrids (**57-65**) were synthesized and purified (Figure 4.2.4.3.2b). Similar to the *N*-nitrosoamine hybrids (**46-48**), the differences between empirical and theoretical microanalytical values of the N^1-substituted diazen-1-ium-1,2-diolate hybrids **57-60** and **63** were greater than 0.4%. Microanalysis was not performed for compounds **61-62**, and **64-65**. The ^{1}H NMR spectra of compounds **57-65** suggested that the samples had only

minute amount of proton-containing impurities (Figure 4.2.4.3.2c). The fragmentation patterns, as determined by positive field electrospray mass spectrometry, confirmed the identities of the compounds.

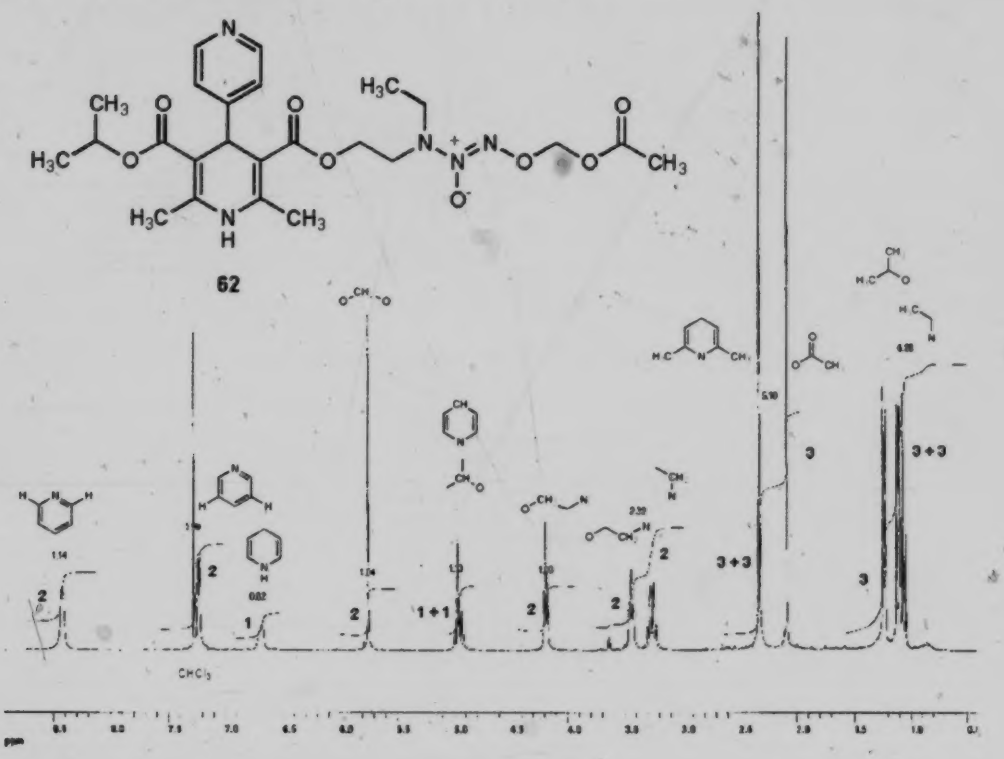


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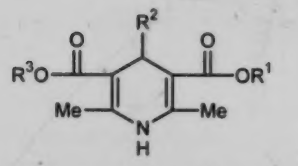
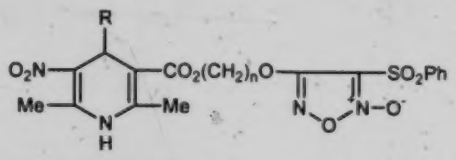
Figure 4.2.4.3.2c: ^{1}H NMR Spectrum of 3-Isopropyl 5-(2-[*N*-(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(4-pyridinyl)-3,5-pyridinedicarboxylate (**62**) in $CDCl_3$ at 22°C

4.3 PHARMACOLOGICAL RESULTS

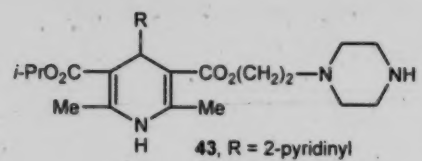
Table 4.3: *In Vitro* Calcium Channel Modulation Activities and Nitric Oxide Release Data for Compounds **6-17**, **24-38**, **43-44**, **46-48**, and **57-65**.



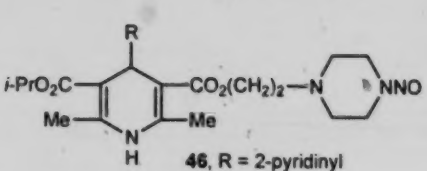
6, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = Et
7, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = *i*-Pr
8, $R^1 = (CH_2)_2ONO_2$; R^2 = 3-pyridinyl; R^3 = *i*-Pr
9, $R^1 = (CH_2)_2ONO_2$; R^2 = 4-pyridinyl; R^3 = *i*-Pr
10, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = *i*-Bu
11, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = *t*-Bu
12, $R^1 = (CH_2)_3ONO_2$; R^2 = 2-pyridinyl; R^3 = *i*-Pr
13, $R^1 = (CH_2)_4ONO_2$; R^2 = 2-pyridinyl; R^3 = *i*-Pr
14, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 2-pyridinyl; R^3 = *i*-Pr
15, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 3-pyridinyl; R^3 = *i*-Pr
16, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 4-pyridinyl; R^3 = *i*-Pr
17, $R^1 = (CH_2)_4Cl$; R^2 = 2-pyridinyl; R^3 = *i*-Pr



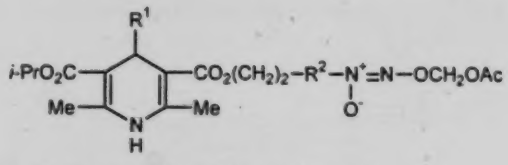
24, R = 2-pyridinyl; n = 2
25, R = 2-pyridinyl; n = 3
26, R = 2-pyridinyl; n = 4
27, R = 3-pyridinyl; n = 2
28, R = 3-pyridinyl; n = 3
29, R = 3-pyridinyl; n = 4
30, R = 4-pyridinyl; n = 2
31, R = 4-pyridinyl; n = 3
32, R = 4-pyridinyl; n = 4
33, R = 2-trifluoromethylphenyl; n = 2
34, R = 2-trifluoromethylphenyl; n = 3
35, R = 2-trifluoromethylphenyl; n = 4
36, R = benzofurazan-4-yl; n = 2
37, R = benzofurazan-4-yl; n = 3
38, R = benzofurazan-4-yl; n = 4



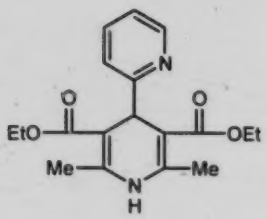
43, R = 2-pyridinyl
44, R = 3-pyridinyl



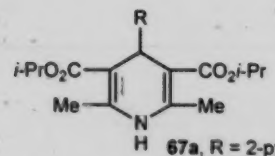
46, R = 2-pyridinyl
47, R = 3-pyridinyl
48, R = 4-pyridinyl



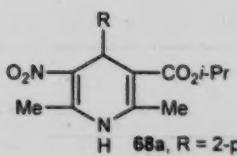
57, R^1 = 2-pyridinyl; R^2 = 1,4-piperazinyl
58, R^1 = 3-pyridinyl; R^2 = NMe
59, R^1 = 4-pyridinyl; R^2 = NMe
60, R^1 = 2-pyridinyl; R^2 = NEt
61, R^1 = 3-pyridinyl; R^2 = NEt
62, R^1 = 4-pyridinyl; R^2 = NEt
63, R^1 = 2-pyridinyl; R^2 = N-(*n*-Bu)
64, R^1 = 3-pyridinyl; R^2 = N-(*n*-Bu)
65, R^1 = 4-pyridinyl; R^2 = N-(*n*-Bu)



66



67a, R = 2-pyridinyl
67b, R = 3-pyridinyl



68a, R = 2-pyridinyl
68b, R = 3-pyridinyl
68c, R = 4-pyridinyl

	GPILSM (μM)		GPLA (μM)	% Nitric Oxide Released (mol/mol)		
	IC_{50}[a]	EC_{50}[b]	EC_{50}[c]	*N*-AcCyst[d]	- / + L-Cyst[e]	Serum[f]
6	5.27 ±0.30	No effect	—	0.07 ±0.01	—	—
7	0.03 ±0.00	No effect	—	0.08 ±0.01	—	—
8	0.06 ±0.01	No effect	—	0.10 ±0.01	—	—
9	0.22 ±0.08	No effect	—	0.09 ±0.01	—	—
10	1.36 ±0.05	No effect	—	0.07 ±0.00	—	—
11	3.14 ±0.46	No effect	—	0.07 ±0.01	—	—
12	0.21 ±0.00	No effect	—	0.03 ±0.00	—	—
13	0.31 ±0.04	No effect	—	0.04 ±0.01	—	—
14	0.15 ±0.01	No effect	—	0.58 ±0.02	—	—
15	0.57 ±0.01	No effect	—	0.84 ±0.02	—	—
16	0.07 ±0.00	No effect	—	0.77 ±0.01	—	—
17	0.21 ±0.03	No effect	—	—	—	—
24	1.44 ±0.25	4.14 ±0.49	15.63 ±6.12	—	1 ±0; 66 ±1	—
25	0.46 ±0.13	> 2.99 (10%)	11.68 ±4.56	—	1 ±0; 70 ±1	—
26	1.11 ±0.03	> 2.99 (13%)	32.69 ±11.82	—	1 ±0; 66 ±2	—
27	5.23 ±1.39	1.89 ±0.83	3.02 ±0.32	—	1 ±0; 74 ±5	—
28	4.42 ±1.24	15.08 ±6.87	19.74 ±10.41	—	1 ±0; 68 ±1	—
29	1.29 ±0.14	7.00 ±2.20	4.06 ±1.41	—	1 ±0; 64 ±1	—
30	1.51 ±0.12	1.44 ±0.25	8.84 ±2.93	—	1 ±0; 72 ±2	—
31	2.45 ±0.45	15.05 ±5.20	7.11 ±2.60	—	1 ±0; 66 ±3	—
32	3.20 ±0.42	4.98 ±1.64	6.71 ±3.49	—	1 ±0; 64 ±1	—
33	> 29.91 (32%)	No effect	5.48 ±0.39	—	7 ±0; 54 ±0	—
34	> 29.91 (48%)	No effect	> 44.66 (34%)	—	9 ±0; 51 ±0	—
35	> 11.96 (40%)	No effect	> 44.66 (10%)	—	7 ±1; 36 ±0	—
36	> 11.96 (46%)	No effect	42.72 ±5.29	—	11 ±1; 60 ±2	—
37	10.04 ±1.50	> 2.99 (12%)	> 44.66 (14%)	—	8 ±1; 58 ±0	—
38	> 29.91 (32%)	> 2.99 (10%)	25.39 ±20.83	—	8 ±0; 50 ±0	—
43	0.26 ±0.04	No effect	—	—	—	—
44	0.47 ±0.09	No effect	—	—	—	—
46	0.29 ±0.02	No effect	—	—	N/A; 2 ±0	—
47	0.22 ±0.01	No effect	—	—	N/A; 1 ±0	—
48	1.16 ±0.16	No effect	—	—	N/A; 1 ±0	—
57	1.19 ±0.08	No effect	—	—	N/A; 2 ±0	141 ±6
58	0.11 ±0.02	No effect	—	—	N/A; 1 ±0	24 ±4
59	0.37 ±0.11	No effect	—	—	N/A; 3 ±0	70 ±8
60	1.28 ±0.15	No effect	—	—	N/A; 3 ±0	132 ±3
61	3.35 ±0.14	No effect	—	—	N/A; 3 ±0	146 ±5
62	0.82 ±0.13	No effect	—	—	N/A; 1 ±0	101 ±4

	GPILSM (μM)		GPLA (μM)	% Nitric Oxide Released (mol/mol)		
	IC_{50}[a]	EC_{50}[b]	EC_{50}[c]	*N*-AcCyst[d]	- / + L-Cyst[e]	Serum[f]
63	0.27 ±0.03	No effect	—	—	N/A; 3 ±0	109 ±3
64	0.73 ±0.22	No effect	—	—	N/A; 2 ±0	109 ±2
65	0.85 ±0.29	No effect	—	—	N/A; 3 ±0	103 ±9
NFD[g]	0.01 ±0.00	—	—	—	—	—
BAYK[g]	—	0.23 ±0.01	0.77 ±0.01	—	—	—
PN[g]	0.04 ±0.01	—	9.40 ±2.60	—	—	—
66[g]	0.18 ±0.00	—	—	—	—	—
67a[g]	0.13 ±0.02	—	—	—	—	—
67b[g]	0.26 ±0.06	—	—	—	—	—
68a[g]	4.87 ±2.06	—	9.67 ±1.27	—	—	—
68b[g]	—	48	28.5 ±2.0	—	—	—
68c[g]	—	35	8.05 ±2.14	—	—	—
GTN[g]	—	—	—	11.43 ±0.63	2 ±0; 20 ±1	1 ±0

[a] The micromolar concentration of the test compound causing a 50% decrease in the slow component or tonic contractile response (IC_{50} ± SEM, n = 3; except for compound **38** where n = 2) in guinea pig ileum longitudinal smooth muscle induced by the muscarinic agonist, carbachol (0.16 μM), was determined graphically from the dose-response curves (Section 3.2.1). For compounds where an IC_{50} value could not be determined, the percent decrease in the contractile response is listed in parentheses at the highest test compound concentration employed.

[b] The micromolar concentration of the test compound causing a 50% increase in the slow component or tonic contractile response (EC_{50} ± SEM, n = 3) in guinea pig ileum longitudinal smooth muscle (in the absence of carbachol) relative to the contraction induced by the reference compound carbachol (0.16 μM), was determined graphically from the dose-response curves (Section 3.2.1). For compounds where an EC_{50} value could not be determined, the percent decrease in the contractile response relative to that produced by carbachol is listed in parentheses at the highest test compound concentration employed. "No effect" denotes that no smooth muscle calcium channel agonist response was observed at the highest test compound concentration employed (44.66 μM).

[c] The micromolar concentration of the test compound causing a 50% increase in the cardiac contractile force (EC_{50} ± SEM, n = 3 except for compounds **36-38** where n = 2) in guinea pig left atrium relative to its basal contractile force, was determined graphically from the dose-response curves (Section 3.2.1). For compounds where an EC_{50} value could not be determined, the percent increase in contractile force at the highest test compound concentration employed is listed in parentheses.

[d] The percent nitric oxide released (± SEM, n = 3) was estimated as the percent nitrite produced per mole of the test compound (2.5 mM) in the presence of *N*-acetylcysteamine (2.5 mM for compounds **6-13**; 5.0 mM for compounds **14-16**) in acetonitrile / phosphate buffer solution (1:1) using the Griess reagent as a dye (Section 3.2.2.1). Numerical values were obtained graphically from a standard nitrite concentration curve.

[e] The percent nitric oxide released (± SEM, n = 3) was estimated as the percent nitrite produced per mole of the test compound (0.1 mM) in the absence (-) or presence (+) of L-cysteine (3.2 mM) in DMSO / phosphate buffer solution (5:95, v/v) for 1 hour at 37°C using the Griess reagent as a dye (Section 3.2.2.2). Numerical values were obtained graphically from a standard nitrite concentration curve. "N/A" denotes that no tests were performed.

[f] The percent nitric oxide released (± SEM, n = 3) was estimated as the percent nitrite produced per mole of the test compound (10 mM) in acetonitrile and guinea pig serum (5:95 v/v) for 1 hour at 37°C using the Griess reagent as a dye (Section 3.2.2.3). Numerical values were obtained graphically from a standard nitrite concentration curve.

[g] "NFD" = nifedipine; "BAYK" = BAY K 8644; "PN" = PN 202 791; "GTN" = glyceryl trinitrate (Figures 1.4.1.4f and 1.5.2a). Pharmacological data for nifedipine, BAY K 8644, PN 202 791 and compounds **68a-c** were taken from the literature [Vo *et al.*, 1995]. Pharmacological data for compound **66** and compounds **67a-b** were taken from the literature [Dagnino *et al.*, 1986].

4.3.1 Nitric Oxide Release

4.3.1.1 *In Vitro* Nitric Oxide Release Assay

In the organic nitrate and furoxan classes of nitric oxide donor compounds (**6-16**, **24-38**), a thiol group is necessary to accelerate the release of nitric oxide [Wang *et al.*, 2002]. *In vitro* studies to determine nitric oxide release were performed in the presence of the thiols *N*-acetylcysteamine and L-cysteine. The percent of nitric oxide released is estimated from the quantification of nitrite using the Griess reaction, because nitric oxide readily oxidizes to nitrite.

The organic nitrate hybrids (**6-16**) were the first class of nitric oxide donor hybrids to be tested by this indirect nitric oxide assay method. The use of *N*-acetylcysteamine in the procedure (Section 3.2.2.1) gave an overall low detection.

Over time, as more literature reports became available, the method was improved. The foul smelling *N*-acetylcysteamine was replaced by L-cysteine, and the amount of non-physiological solvent was reduced from 50% (acetonitrile) to 5%

(dimethyl sulfoxide) (v/v). With the *N*-acetylcysteamine method (Section 3.2.2.1), glyceryl trinitrate was determined to release 11% nitric oxide, whereas the L-cysteine method indicated 20%. Comparatively, the L-cysteine method marked an almost two-fold improvement in nitrite detection.

To test the dependency of the L-cysteine method, single point studies (n = 1) were performed at three different incubation times (1, 2 and 3 hours).

1. Does the Griess reagent give false positives over time?

No. The 3 hour incubation of a blank buffer solution only showed 0.3% higher nitrite detection level than that for the 1 hour incubation.

2. Does the presence of 5% DMSO give a false positive?

No. After three hours of incubation, the presence of 5% DMSO produced a 0.5% higher level of nitrite detection than that for the blank buffer solution.

3. Does the concentration of excess L-cysteine (1.7 mM, 3.3 mM, 5.0 mM) relative to the test compound (0.1 mM: glyceryl trinitrate, compound **29**) affect nitrite detection?

Yes. After the 1 hour incubation, glyceryl trinitrate was detected at 0, 11, 22 and 33% at 0, 1.7, 3.3 and 5.0 mM respectively with a linear increase of 0, 3, 5 and 7% nitric oxide released per hour, respectively (Figure 4.3.1.1.). After the 1 hour incubation, the percent nitric oxide release from compound **29** was detected at 1, 52, 62 and 72% at 0, 1.7, 3.3 and 5.0 mM respectively with a linear increase of 0, 1, 1 and 1% nitric oxide released per hour respectively. Thus, a higher concentration of excess L-cysteine gave higher nitrite detection. Moreover, the incubation time is also significant in that the rate of increase of nitric oxide released is different for different compounds. For example, when given enough time, the percent of nitrite detected after the release of nitric oxide from glyceryl trinitrate can be higher than that from compound **29**. To avoid these effects, compounds **24-38** were tested using the same Griess reagent, the same mixture of L-cysteine in buffer solution, and the time intervals were strictly followed.

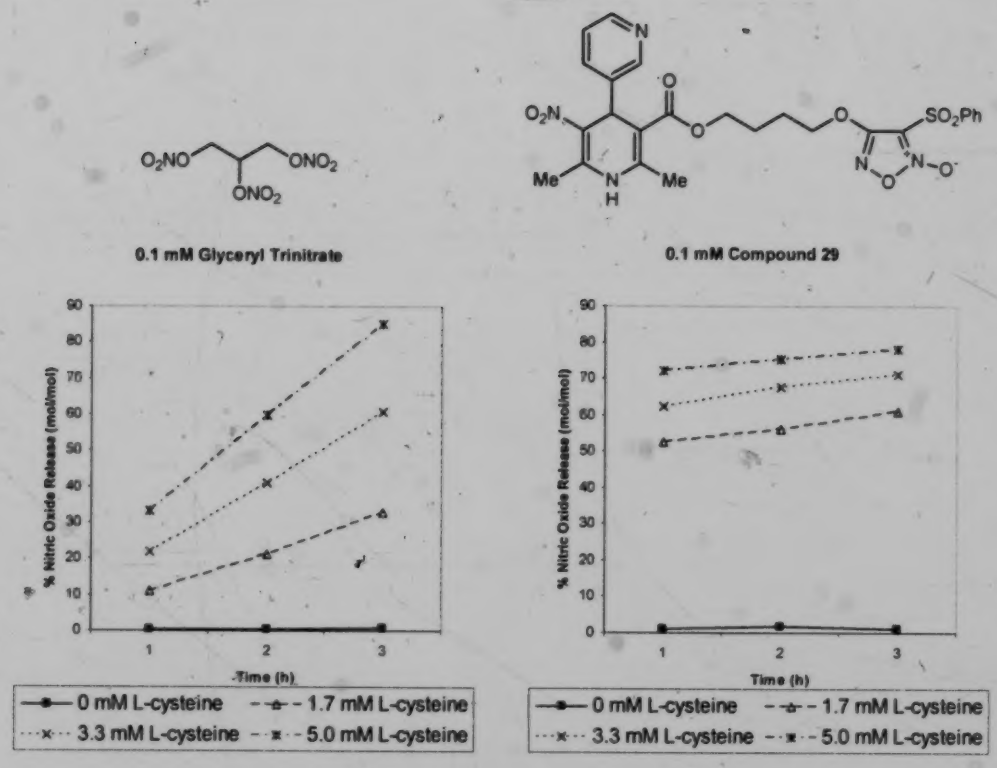


Figure 4.3.1.1: The Percent Nitric Oxide Release from Glyceryl Trinitrate and Compound **29** (0.1 mM) in Solutions Containing Different Concentrations of L-Cysteine over Time.

When the furoxan hybrids (**24-38**) were tested, L-cysteine (3.4 mM) in buffer solution was prepared by first adding L-cysteine (5 mM) to a phosphate buffer solution (50 mN) and titrating the mixture with KH_2PO_4(aq) (50 mN) until pH 7.4. The volume of KH_2PO_4(aq) added during the titration diluted the L-cysteine concentration to 3.4 mM (Section 3.2.2.2). In later experiments with the L-cysteine method (**46-48** and **57-65**), we discovered that a better technique for keeping the L-cysteine concentration consistent at 3.4 mM between experiments is to titrate a basic solution of L-cysteine (3.4 mM) in K_2HPO_4(aq) (50 mN) with an acidic solution of L-cysteine (3.4 mM) in KH_2PO_4(aq) (50 mN).

The N^1-substituted diazen-1-ium-1,2-diolate hybrids (**57-65**) were tested for nitric oxide release by the L-cysteine method (Section 3.2.2.2). No nitric oxide was released because the N^1-substituted diazen-1-ium-1,2-diolates were protected by an ester moiety.

Incubation with guinea pig esterases (serum) to liberate the nitric oxide donor moiety was carried out. A multiple-cuvettes plate ultraviolet spectrophotometer (Thermo Labsystem Multiscan Ascent) was used in place of a single cuvette reader (Phillips PU 8740 UV/VIS scanning spectrophotometer) to minimize the amount of guinea pig serum required to perform the assays. Furthermore, the assays performed using the multiple-cuvettes plate reader were less time consuming and permitted more stringent time control. However, the experimental errors increased, because smaller volumes (100 µM range), than previous (1.5 mL), were used. Nitric oxide released from compounds **57-65** dissolved in DMSO and guinea pig serum (5:95 v/v) was determined. The nitric oxide released as nitrite was fairly low (ranging from 1 to 13%, n = 3, data not shown). These assays were repeated using acetonitrile (5:95 v/v) in place of dimethyl sulfoxide, because dimethyl sulfoxide may have denatured the guinea pig esterases. The repeated assays showed higher extents of nitric oxide release (Table 4.3).

4.3.1.2 Organic Nitrate Hybrids

The percent nitric oxide released *in vitro* from the organic nitrate hybrids (**6-16**) (range of 0.03 – 0.84%) was lower than that for the reference drug, glyceryl trinitrate (11.43%).

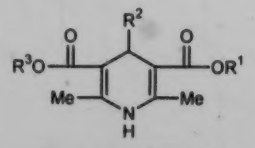
The number of nitrooxy groups present affects the extent of nitric oxide released and this is attributed to strain differences due to interactions of the nitrooxy groups within the C-5 substituents [Curry & Aburawi, 1985; Fung, 1992; Seth & Fung, 1993]. In our experiments, glyceryl trinitrate released 11.43%, the dinitrates (**14-16**) released 0.58 – 0.84%, and the mononitrates (**6-13**) released 0.03 – 0.10% nitric oxide per molecule of the test compound (trinitrate >> dinitrate > mononitrate). These data support the concept that steric strain facilitates nitric oxide release from organic nitrates.

Gasco *et al.* (1996) noted that the *in vitro* vasodilating activity of aliphatic nitrates is linearly dependent on lipophilicity. However, the extent of nitric oxide released from the mononitrates **6-13** may, at least in part, have an inverse dependent relationship to the length of the nitrooxyalkyl chain [$-(CH_2)_2ONO_2$ (**7**) > $-(CH_2)_3ONO_2$ (**12**) and $-(CH_2)_4ONO_2$ (**13**)].

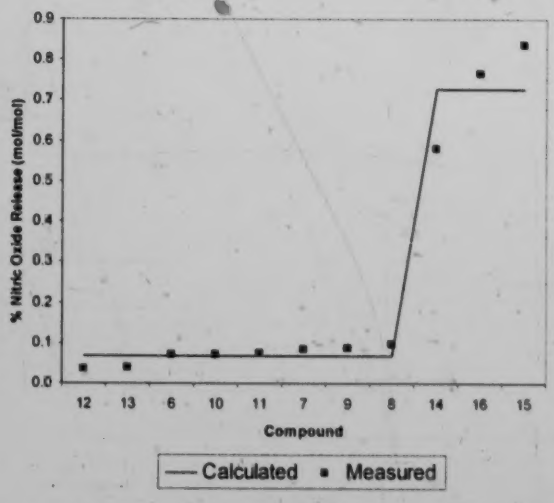
Steric interactions between the C-3, C-4 and C-5 substituents may also be a partial determinant of nitric oxide release, since the nitric oxide release order for the dinitrate isomers was 3-pyridinyl (**15**) > 4-pyridinyl (**16**) > 2-pyridinyl (**13**) which correlates inversely with the interspatial distance between the respective pyridinyl nitrogen atom and the closest oxygen atom of the nearest nitrooxy group (4.49 Å, 5.29 Å and 7.07 Å, respectively) as measured from the PM3 geometry optimized structures. Although the differences in nitric oxide release were very small, a similar trend appears to exist for the related $-CH_2CH_2ONO_2$ analogues (**7-9**). Hence, one could speculate that the proximity of the pyridinyl nitrogen may induce a destabilization of the nitrooxy group, which may enhance the release of nitric oxide.

Substitution on the non-nitrooxy ester side of the 1,4-dihydropyridine ring suggests a minor contribution to nitric oxide release of the pattern *i*-Pr (**7**) > Et (**6**), *i*-Bu (**10**) and *t*-Bu (**11**).

Table 4.3.1.2: A Quantitative-Structure Activity Relationship Equation for the Extent of Nitric Oxide Released from 3-Nitrooxyalkyl 5-Alkyl 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**6-17**)



6, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = Et
7, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = *i*-Pr
8, $R^1 = (CH_2)_2ONO_2$; R^2 = 3-pyridinyl; R^3 = *i*-Pr
9, $R^1 = (CH_2)_2ONO_2$; R^2 = 4-pyridinyl; R^3 = *i*-Pr
10, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = *i*-Bu
11, $R^1 = (CH_2)_2ONO_2$; R^2 = 2-pyridinyl; R^3 = *t*-Bu
12, $R^1 = (CH_2)_3ONO_2$; R^2 = 2-pyridinyl; R^3 = *i*-Pr
13, $R^1 = (CH_2)_4ONO_2$; R^2 = 2-pyridinyl; R^3 = *i*-Pr
14, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 2-pyridinyl; R^3 = *i*-Pr
15, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 3-pyridinyl; R^3 = *i*-Pr
16, $R^1 = CH(CH_2ONO_2)_2$; R^2 = 4-pyridinyl; R^3 = *i*-Pr



% NO Release = A + 0.069

n = 11; R^2 = 0.96; $p < 0.001$

If the nitric oxide donor is a mononitrate, A = 0.000.

If the nitric oxide donor is a dinitrate, A = 0.659.

A multiple-collinear regression equation can be formulated by parameterizing the above observations: the number of nitrooxy groups, the length of the nitrooxyalkyl chain, the point of attachment of the pyridinyl ring, and the "essential side" alkyl substitution.

The equation was derived as followed. In the case of the "number of nitrooxy group" parameter, mononitrates were originally given a score of 0.069 (the average of the results for compounds **6-13**), while dinitrates were scored at 0.727 (the average of the results for compounds **14-16**). The difference (w_{1a} = 0.727 – 0.069 = 0.659) between the dinitrates and monitrates scores indicated the weight of the parameter in the equation. The original scores were adjusted to values between 0 (the lowest original score) and 1 (the highest original score) relative to w_{1a}. In this case, the mononitrates had a score of 0 and dinitrates had a score of 1. The same types of calculation were performed for the other parameters: the length of the nitrooxyalkyl chain, the point of attachment of the pyridinyl ring, and the "essential side" alkyl substitution. The weights of the parameters (w_{1a}, w_{1b}, w_{1c}, w_{1d}) were adjusted to values between 0 (the lowest weight) and 1 (the highest weight), which gave rise to (w_{2a} = 1.000; w_{2b} = 0.065; w_{2c} = 0.046; w_{2d} = 0.000). The percentile of the adjusted weight (w_2) of a parameter indicated its contribution to the equation and also the extent of its effect on the other parameters. In the situation where multiple values could be assigned as the original score, the lowest score was used since it represented a result which was least affected by the other parameters. For example, in determining the score for an isopropyl substituent, the average of compounds **6-9** and **12-13** (0.068) was chosen over the average of compounds **14-16** (0.727). Lastly, the scores were weighed by multiplying their current values with their respective weight (w_1).

A multiple collinear regression equation was derived using Microsoft-Excel (2003) (Table 4.3.1.2). Although Property-Intrinsic Activity Relationship equation could be derived from all four parameters, the adjusted weights indicated that "the number of nitrooxy group" is by far the strongest determinant. An equation that considered all four parameters had a coefficient of determination (R^2) of 0.98 (data not shown). An equation that only considered the "number of nitrooxy group" parameter had a coefficient of determination of 0.96. A quantitative structure-activity relationship equation is considered valid when it has the least number of parameters. In this case, reducing four parameters to one parameter did not greatly influence the coefficient of determination. Hence, the single parameter equation is comparatively more valid than the four parameter equation.

The best test compound (**15**) only ranks as the 7th percentile relative to glyceryl trinitrate. Indeed, the test compounds (**6-16**) are considered to be low nitric oxide donors and do not entice further studies.

4.3.1.3 Nitric Oxide Donor Furoxan Hybrids

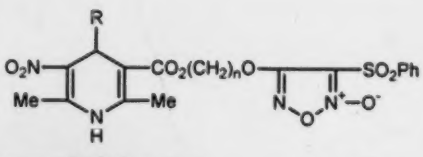
The percent nitric oxide released *in vitro* from the furoxan hybrids (**24-38**) (range of 36 – 74%) was higher than that for the reference drug, glyceryl trinitrate (20%). The best test compound (**27**) released 3.7-fold more nitric oxide than glyceryl trinitrate.

The percent nitric oxide release, in the absence of L-cysteine, was much lower (1 – 11 % range) than that in the presence of 3.2 mM L-cysteine (36 – 74%). These results are consistent with reports that buffer containing reduced thiols such as L-cysteine or glutathione, which serve as a source of thiols equivalent to the mercapto groups present in plasma are required for the release of nitric oxide from a furoxan moiety [Feelisch *et al.*, 1992; Civelli *et* al., 1994; Medana *et al.*, 1994; Ferioli, *et al.*, 1995; Civelli *et al.*, 1996; Medana *et al.*, 1999]. In this regard, it is interesting that the C-4 2-trifluoromethylphenyl (**33-35**), and 4-benzofurazanyl (**36-38**), compounds that released nitric oxide more effectively in the absence of L-cysteine (7 – 11% range), relative to the related C-4 pyridinyl compounds (**24-32**) that released a negligible amount of nitric oxide (1%), do not induce an agonist contractile effect (**33-35**) or induce a modest increase in guinea pig ileum longitudinal smooth muscle contractility (**36-38**) in the absence of carbachol (Section 4.3.2.2.2). Moreover, the addition of L-cysteine to the calcium channel antagonist assay organ bath in the absence of carbachol abolishes the undesired contractile effect. This implies that nitric oxide may counter-act the undesirable smooth muscle contraction of calcium channel agonists. The results of this study support the hypothesis that the calcium channel modulation (agonist / antagonist) effects of hydrid compounds, designed to simultaneously release nitric oxide, provides a useful drug design concept.

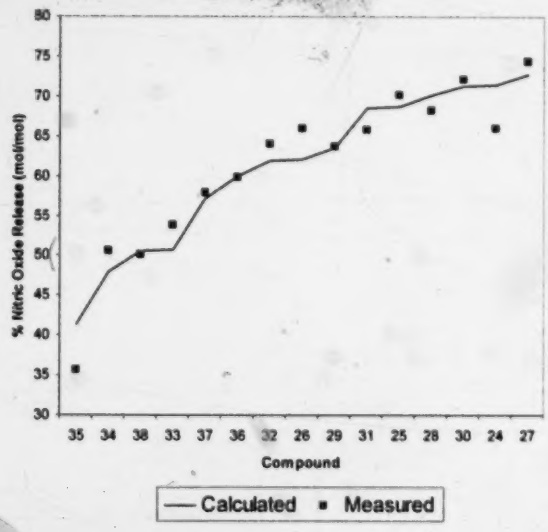
A multiple collinear regression equation was derived from the nitric oxide release data for compounds **24-38** (Table 4.3.1.3) following the method described in Section 4.3.1.2. As the equation suggests, the extent of nitric oxide released is mainly determined by C-4 substitution which is ranked as 3-pyridinyl ≥ 2-, 4-pyridinyl > 2-trifluoromethylphenyl > 4-benzofurazanyl, which suggests that nitric oxide release is inversely proportional to the size of the C-4 substitution. Lengthening the alkyl-chain where the furoxan group is attached decreases the extent of nitric oxide released.

In a different study, our research group has attached furoxan moieties to nucleosides as potential hybrid nitric oxide donor-nucleoside anticancer agents (Figure 1.5.3e). The resulting compounds were extremely cytotoxic to both normal and cancer cell lines. This data suggest that the *in vivo* toxicity of furoxan nitric oxide donor compounds toward normal cell lines may represent an obstacle to their potential clinical use [Moharram *et al.*, 2004]. In light of this, we were not compelled to pursue further studies with furoxans.

Table 4.3.1.3: A Quantitative-Structure Activity Relationship Equation for the Extent of Nitric Oxide Released from 5-[(3-Benzenesulfonyl)furoxan-4-yloxy]alkyl 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl, benzofurazan-4-yl, 2-, 3- or 4-pyridinyl)-5-pyridinecarboxylates (**24-38**)



24, R = 2-pyridinyl; n = 2
25, R = 2-pyridinyl; n = 3
26, R = 2-pyridinyl; n = 4
27, R = 3-pyridinyl; n = 2
28, R = 3-pyridinyl; n = 3
29, R = 3-pyridinyl; n = 4
30, R = 4-pyridinyl; n = 2
31, R = 4-pyridinyl; n = 3
32, R = 4-pyridinyl; n = 4
33, R = 2-trifluoromethylphenyl; n = 2
34, R = 2-trifluoromethylphenyl; n = 3
35, R = 2-trifluoromethylphenyl; n = 4
36, R = benzofurazan-4-yl; n = 2
37, R = benzofurazan-4-yl; n = 3
38, R = benzofurazan-4-yl; n = 4



% NO Release = R + N + 41.370

n = 15; R^2 = 0.92; p < 0.001

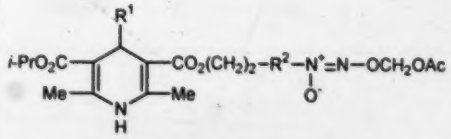
R		N	
3-pyridinyl	22.083	n = 2	9.310
2-pyridinyl	20.717	n = 3	6.630
4-pyridinyl	20.583	n = 4	0.000
2-trifluoromethylphenyl	9.217		
4-benzofurazanyl	0.000		

4.3.1.4 *N*-Nitrosoamine Hybrids

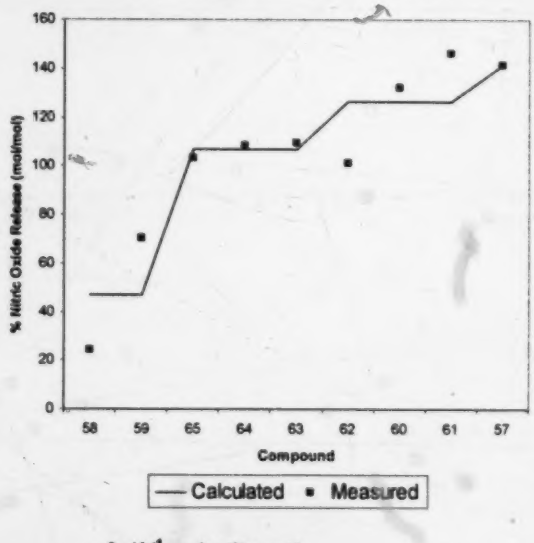
Contrary to the supposition that *N*-nitrosoamines release nitric oxide [Wang *et al.* (2002)], none of the 3-isopropyl 5-(2-[4-nitrosopiperazinyl]ethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**46-48**) prepared in this study released nitric oxide.

4.3.1.5 N^1-Substituted Diazen-1-ium-1,2,-diolate Hybrids

Table 4.3.1.5: A Quantitative-Structure Activity Relationship Equation for the Extent of Nitric Oxide Released from 5-(2-[(O^2-Acetoxymethyldiazen-1-ium-1,2-diolate)(*N*,*N*-dialkylamino or 4-piperazin-1-yl)]ethyl) 1,4-Dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**57-65**)



57, R^1 = 2-pyridinyl; R^2 = 1,4-piperazinyl
58, R^1 = 3-pyridinyl; R^2 = NMe
59, R^1 = 4-pyridinyl; R^2 = NMe
60, R^1 = 2-pyridinyl; R^2 = NEt
61, R^1 = 3-pyridinyl; R^2 = NEt
62, R^1 = 4-pyridinyl; R^2 = NEt
63, R^1 = 2-pyridinyl; R^2 = N-(*n*-Bu)
64, R^1 = 3-pyridinyl; R^2 = N-(*n*-Bu)
65, R^1 = 4-pyridinyl; R^2 = N-(*n*-Bu)



% NO Release = A + 47.160

n = 9; R^2 = 0.81; p = 0.001

A (N^1-substituent)	
1,4-piperazinyl	94.070
N-ethyl	79.223
N-(*n*-butyl)	59.833
N-methyl	0.000

The percent nitric oxide released in serum from the N^1-substituted diazen-1-ium-1,2-diolate hybrids (**57-65**) (range of 24 – 141%) was higher than that for the reference drug, glyceryl trinitrate (1%). Guinea pig serum does not contain sufficient L-cysteine to

enhance the release of nitric oxide. The best test compound (**61**) released 7.2-fold more nitric oxide than glyceryl trinitrate in the presence of L-cysteine (20%).

A multiple collinear regression equation was derived from the nitric oxide release data for compounds **57-65** (Table 4.3.1.5) following the method described in Section 4.3.1.2. According to Hrabie & Keefer (2002), the rate of nitric oxide release from the N^1-substituted diazen-1-ium-1,2-diolate moiety can be controlled by the nature of the N^1-side-chain. In the group of compounds **57-65**, substitution at the N^1-side chain is a determinant of the extent of nitric oxide released following the general order of 1,4-piperazinyl > *N*-Et > *N*-(*n*-Bu) >> *N*-Me. Including the pyridinyl C-4 substituents as a parameter decreases the validity of the equation, which suggests that there are no clear trends between C-4 2-, 3- or 4-pyridinyl substituents. Unfortunately, the equation is not very reliable, because the sample size is small (n = 9) and the equation is based on four scores.

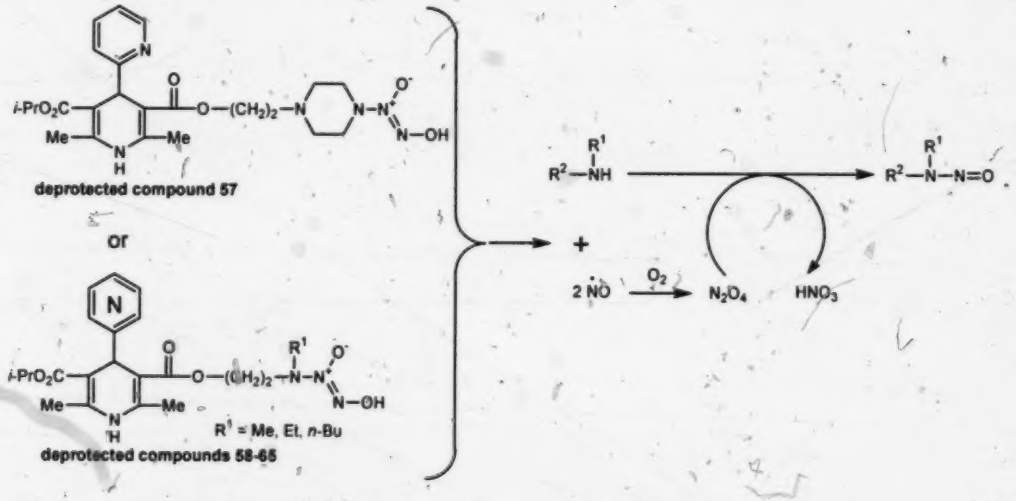


Figure 4.3.1.5: The Release of Nitric Oxide from the N^1-Substituted Diazen-1-ium-1,2,-diolate Hybrids Synthesized (**57-65**)

During the process of nitric oxide release, the protective acetoxymethyl group of the parent compound (**57-65**) is removed by esterases found in guinea pig serum (Figure 4.3.1.5). Two molecules of nitric oxide are liberated from the parent compound [Keefer, 1998]. One nitric oxide molecule is recaptured by the amine product.

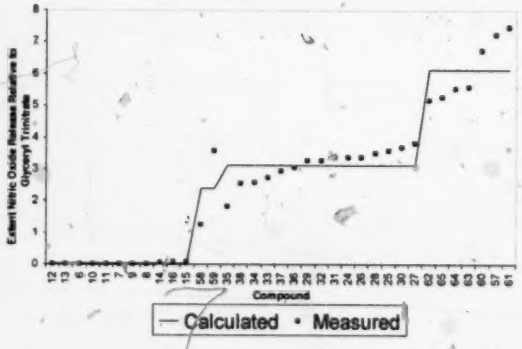
Interestingly, a fair number of diazen-1-ium-1,2-diolate hybrids (**57** and **60-65**) have nitrite detection greater than 100% which suggests that the rate of conversion from the amine post-cursor to *N*-nitrosoamine is slower than the rate of nitric oxide release. The results for the *N*-nitrosoamine hybrids (**46-48**) indicate that *N*-nitrosoamines do not give false positives in the Griess nitrite detection assay.

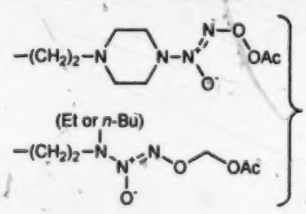
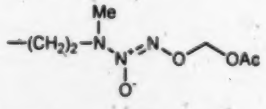
4.3.1.6 An Overall Quantitative Structure-Activity Relationship Equation Correlating the Structures of the Compounds Synthesized with Nitric Oxide Release

Table 4.3.1.6: A Quantitative-Structure Activity Relationship Equation for the Extent of Nitric Oxide Released from Compounds **6-16**, **24-38**, and **57-65**

(Extent of Nitric Oxide Release Relative to Glyceryl Trinitrate) = A + 0.022

$n = 35$; $R^2 = 0.93$; $p < 0.001$



A (nitric oxide donor moiety)	
	6.111
furoxan moiety	3.101
	2.384
organic nitrates	0.000

A multiple collinear regression equation was derived from the nitric oxide release data for nitric oxide donating compounds **6-16**, **24-38** and **57-65** (Table 4.3.1.6) following the method described in Section 4.3.1.2. The nitric oxide release data were ranked as percentiles relative to the amount of nitric oxide released by glyceryl trinitrate, because each method of determining nitric oxide release (Section 3.2.2) had different accuracies. Glyceryl trinitrate was ranked as "1". In the cases of the N^1-substituted diazen-1-ium-1,2,-diolate hybrids (**57-65**), the nitric oxide release data were ranked relative to the

percent of nitric oxide released from glyceryl trinitrate in the presence of L-cysteine (20% nitric oxide release [mol / mol]).

An equation that employs a single parameter with three scores representing the nitrooxy, furoxan, and N^1-substituted diazen-1-ium-1,2-diolate moieties, has a coefficient of determination (R^2) of 0.79 (data not shown). The overall order of the extent of nitric oxide release is as follows: N^1-substituted diazen-1,2-diolate > furoxan > organic nitrate hybrids. The linear-fit of the equation drastically improves when each subtype of nitric oxide donors is evaluated ($R^2 = 0.94$) (data not shown). The equation becomes more valid when related moieties with similar scores are simplified to one score without greatly affecting the coefficient of determination ($R^2 = 0.93$) (Table 4.3.1.6).

4.3.2 Calcium Channel Modulatory Activity

4.3.2.1 Does Nitric Oxide Release Affect Calcium Channel Modulatory Actions?

1,4-Dihydropyridine calcium channel antagonists are potent vasodilators. Nitric oxide donors are also potent vasodilators. A hybrid drug composed of a calcium channel antagonist and a nitric oxide donor may therefore be an even more potent vasodilator.

There is no clear difference in calcium channel antagonist activity between the organic nitrate hybrid **13** (IC_{50} = 0.31 μM) and its non-organic nitrate equivalent compound **17** (IC_{50} = 0.21 μM) (Figure 4.3.2.1). Compared to the other nitric oxide donors, the organic nitrate hybrid **13** is a relatively weak nitric oxide donor (3rd percentile relative to glyceryl trinitrate). Thus, it is proposed that the amount of nitric oxide released is so small, that any synergistic smooth muscle relaxing effect attributed to nitric oxide is negligible. Indeed, the Canadian Pharmacists Association (2003) considers mononitrates to be therapeutically inactive.

A similar relationship exists between the *N*-nitrosoamine hybrids (**46-47**) (IC_{50} = 0.22 – 0.29 μM) and their respective precursors (**43-44**) (IC_{50} = 0.26 – 0.47 μM), because the hybrids (**46-47**) do not release nitric oxide.

A comparison of the calcium channel antagonist activites of 3-isopropyl 5-(2-[4-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)piperazin-1-yl]ethyl) 1,4-dihydro-2,6-dimethyl-4-(2-pyridinyl)-3,5-pyridinedicarboxylate (**57**) (IC_{50} = 1.19 μM) with its piperazinyl (**43**) (IC_{50} = 0.26 μM) and *N*-nitrosoamine (**46**) (IC_{50} = 0.29 μM) analogues,

suggests that nitric oxide released is detrimental to calcium channel antagonist activity. However, calcium channel antagonist assays were performed in the absence of esterases; and thus, very little nitric oxide was released during the *in vitro* assays. Hence, the released nitric oxide is not likely responsible for the weak calcium channel antagonist activity exhibited by the diazen-1-ium-1,2-diolate hybrid **57**.

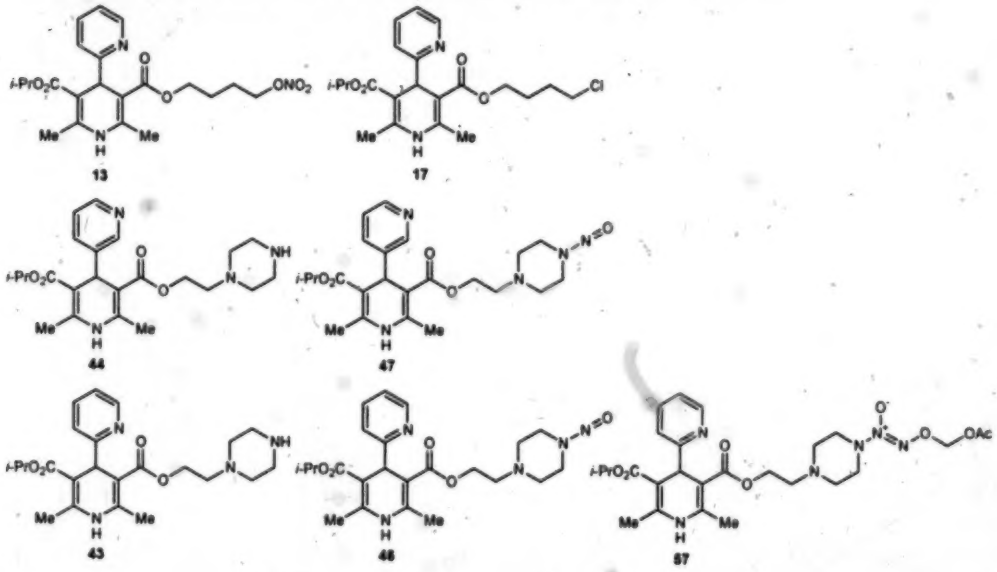


Figure 4.3.2.1: The Nitric Oxide Donor-1,4-Dihydropyridine Hybrids (**13**, **17**, **43-44**, **46-47** and **53**) that are Used to Determined if Nitric Oxide Release Affects Calcium Channel Antagonist Activity

However, as discussed in Section 4.3.1.3 (and will be further elaborated in Section 4.3.2.2.2), the presence of nitric oxide seems to diminish (or abolish) the undesired contractile effect on smooth muscle induced by 1,4-dihydropyridine calcium channel agonists (**24-38**). One possible explanation is that the nitric oxide's smooth muscle relaxing effect is counter-acting the 1,4-dihydropyridine agonist's contractile effect. If this explanation is valid, then it is plausible to assume that nitric oxide and 1,4-dihydropyridine antagonists provide a synergistic smooth muscle relaxing effect. Indeed, it has been shown in combinatory applications that calcium channel antagonists enhance the effect of nitric oxide in vascular smooth muscles [Luscher & Zang, 1993], that the vasodilating effect induced by calcium channel antagonists is increased by nitric oxide donor drugs [Liu *et al.*, 1994; Dhein *et al.*, 1995], and that the combined effects of

basal nitric oxide release and calcium channel antagonists produce an inhibition greater than additive which increases the observed calcium channel antagonist potency by 3-fold [Salomone *et al.*, 1996].

A multiple collinear regression equation relating to calcium channel antagonist activity will be discussed in Section 4.3.2.3. The equation derived will reveal that the nitric oxide released contributes to the enhancement of smooth muscle relaxation.

4.3.2.2 Qualitative Observations Pertaining to Calcium Channel Modulatory Activity for the Hybrid Compounds Investigated

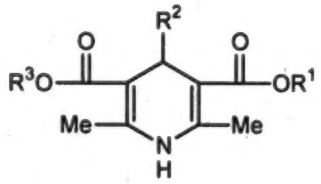
All the 1,4-dihydropyridines calcium channel antagonists synthesized (**6-17**, **24-38**, **43-44**, **46-48**, and **57-65**) exhibited less potent calcium channel antagonist activity (IC_{50} = 0.03 μM to > 29.91 μM) than the reference drug, nifedipine (IC_{50} = 0.01 μM). A variety of substituents at the C-3 (Et, *i*-Pr, *i*-Bu, *t*-Bu), C-4 (2-, 3- or 4-pyridinyl, 2-trifluoromethylphenyl, 4-benzofurazanyl) and C-5 positions (nitrooxy, furoxan, piperazine, nitrosopiperazine, N^1-substituted diazen-1-ium-1,2-diolate moieties) of the 1,4-dihydropyridine ring were investigated to determine their effect on calcium channel antagonist activity.

Most of the 1,4-dihydropyridine calcium channel agonists synthesized (**27-32**, **37-38**), with the exception of the 2-trifluoromethyl compounds **33-35** and **36**, were non-cardioselective in that they induced undesired contractions of guinea pig ileum longitudinal smooth muscles. However, none of the furoxan hybrids (**27-38**) displayed any significant increase in heart rate (less than 20% increase). Thus, reflex tachycardia was not significantly induced by these compounds (**27-38**).

4.3.2.2.1 Organic Nitrate Hybrids

When C-4 2-pyridinyl and C-3 *i*-Pr substituents are present, calcium channel antagonist activity is inversely dependent upon the length of the C-3 nitrooxyalkyl ester substituent where the potency order is $-(CH_2)_2ONO_2$ (**7**) > $-CH(CH_2ONO_2)_2$ (**14**) ≥ $-(CH_2)_3ONO_2$ (**12**) ≥ $-(CH_2)_4ONO_2$ (**13**) (Figure 4.3.2.2.1a). A comparison of the approximately equipotent compounds **13** [$R^1 = (CH_2)_4ONO_2$] and **17** [$R^1 = (CH_2)_4Cl$] suggests the nitrooxy group does not contribute to the calcium channel antagonist activity exhibited by compound **13** (Figure 4.3.2.21b). This observation is also consistent with a comparison to 3,5-dialkyl analogues reported previously [Dagnino *et*

al., 1986] with the corresponding nitrooxylalkyl analogues prepared in this study such as compound **66** with compound **6**, compound **67a** with compound **14**, and compound **67b** with compound **15** (Figure 4.3.2.2.1b). It was concluded that the nitrooxy group does not contribute to calcium channel antagonist activity because very litte nitric oxide is released.



R^1 : $-(CH_2)_2ONO_2 > -CH(CH_2ONO_2)_2 > -(CH_2)_3ONO_2 > -(CH_2)_4ONO_2, -(CH_2)_4Cl$

R^2 : 2-pyridinyl > 3-pyridinyl > 4-pyridinyl

R^3 : *i*-Pr >> Et, *i*-Bu, *t*-Bu

Figure 4.3.2.2.1a: Observations Pertaining to the Calcium Channel Antagonist Potency for the Organic Nitrate Hybrids Synthesized (**6-17**)

A comparison of the effect which the point of attachment of isomeric C-4 pyridinyl substituents has on calcium channel antagonist activity, when C-3 *i*-Pr and C-5 $CH_2CH_2ONO_2$ substituents are present, shows the relative activity order is 2-pyridinyl (**7**) ≥ 3-pyridinyl (**8**) > 4-pyridinyl (**9**) (Figure 4.3.2.2.1a). This activity profile is consistent with results from a previous study employing related 3,5-dialkyl analogues [Dagnino *et al.*, 1986], and well documented structure-activity correlations showing that the relative potency order of a C-4 phenyl ring substituent is generally *ortho* > *meta* > *para* [Fossheim *et al.*, 1982; Bossert & Vater, 1989; Mager *et al.*, 1992; Triggle, 1992]. In contrast, when a sterically larger C-3 $-CH(CH_2ONO_2)_2$ substituent is present, the potency sequence is altered where 4-pyridinyl (**16**) > 2-pyridinyl (**14**) and 3-pyridinyl (**15**). These results suggest that the size of the C-3 and C-5 ester moieties provide interdependent steric contributions to the 1,4-dihydropyridine receptor binding, and hence calcium channel antagonist activity. This interdependency often complicates quantitative structure-activity relationship equations used to correlate calcium channel antagonism [Mager *et al.*, 1992].

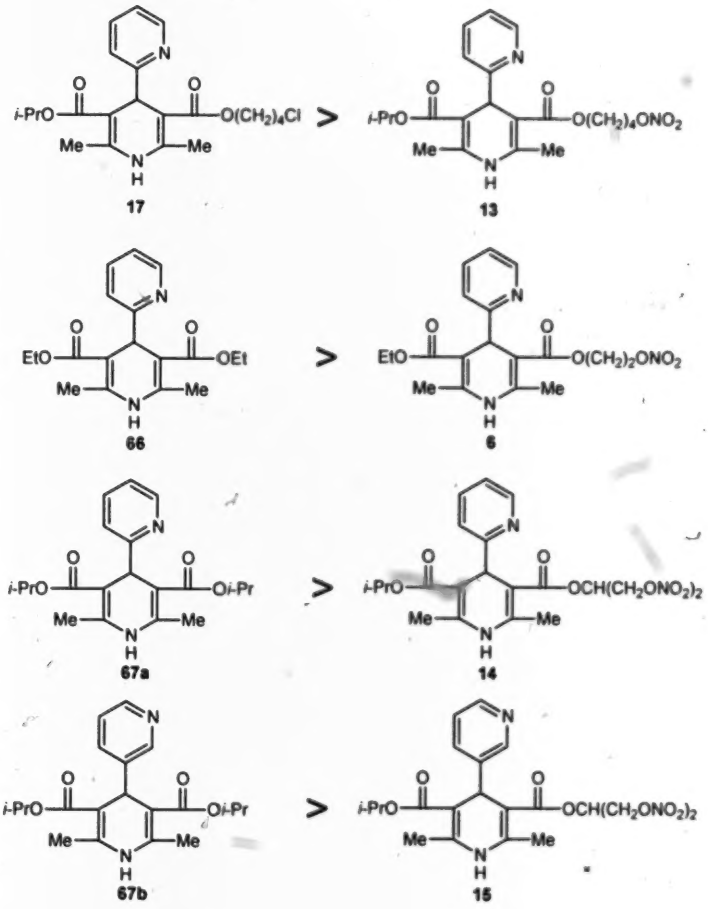


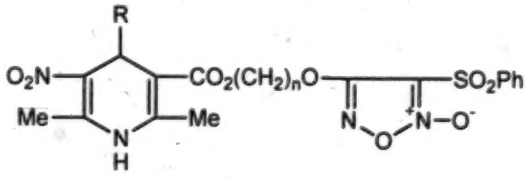
Figure 4.3.2.2.1b: The 1,4-Dihydropyridines (**6**, **13-15**, **17** and **66-67**) that are Used to Determined if Nitric Oxide Released from Nitrooxy Hybrids Affects Calcium Channel Antagonist Activity

Compounds having C-5 $-CH_2CH_2ONO_2$ and C-4 2-pyridinyl substituents show a potency profile, with respect to the C-3 alkyl substituent, of *i*-Pr (**7**) >> the approximately equipotent Et (**6**), *i*-Bu (**10**) and *t*-Bu (**11**) analogues (Figure 4.3.2.2.1a).

4.3.2.2.2 Furoxan Hybrids

Furoxan hybrids possessing a C-4 2-pyridinyl (**24-26**), 3-pyridinyl (**27-29**) or 4-pyridinyl (**30-32**) substituent are more potent calcium channel antagonists (IC_{50} = 0.46 to

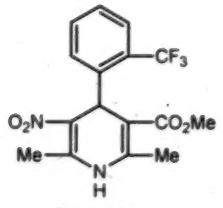
5.23 μM) than related analogues having a C-4 2-trifluoromethylphenyl (**33-35**) (IC_{50} > 44.66 μM), or 4-benzofurazanyl (**36-38**) ($IC_{50} \geq 10.04$ μM) substituent that exhibit modest antagonist activity compared to the reference drug, nifedipine (IC_{50} = 0.01 μM) (Figure 4.3.2.2.2a). The observation that the subgroups of compounds **24-32** having isomeric C-4 2-, 3- and 4-pyridinyl substituents, and alkyl spacer groups of variable chain length [$-CO_2(CH_2)_nO-$, n = 2 – 4] exhibit small differences in potency, indicates that the point of attachment of the C-4 pyridinyl ring and the length of the alkyl spacer are not determinants of calcium channel antagonist activity.



R : (pyridinyl) > 2-trifluoromethylphenyl and 4-benzofurazanyl

n = 2, 3, 4

Figure 4.3.2.2.2a: Observations Pertaining to the Calcium Channel Antagonist Potency for the Furoxan Hybrids Synthesized (**24-38**)



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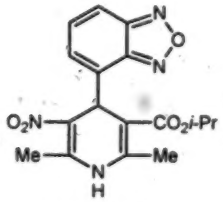
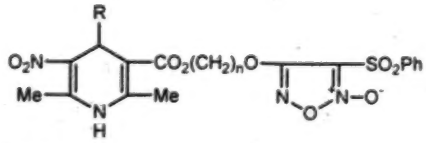
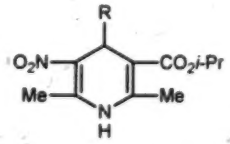


PN 202 791



24, R = 2-pyridinyl; n = 2
25, R = 2-pyridinyl; n = 3
26, R = 2-pyridinyl; n = 4
27, R = 3-pyridinyl; n = 2
28, R = 3-pyridinyl; n = 3
29, R = 3-pyridinyl; n = 4
30, R = 4-pyridinyl; n = 2
31, R = 4-pyridinyl; n = 3
32, R = 4-pyridinyl; n = 4
33, R = 2-trifluoromethylphenyl; n = 2
34, R = 2-trifluoromethylphenyl; n = 3
35, R = 2-trifluoromethylphenyl; n = 4
36, R = benzofurazan-4-yl; n = 2
37, R = benzofurazan-4-yl; n = 3
38, R = benzofurazan-4-yl; n = 4



68a, R = 2-pyridinyl
68b, R = 3-pyridinyl
68c, R = 4-pyridinyl

Figure 4.3.2.2.2b: 1,4-Dihydropyridine Calcium Channel Agonists (BAY K 8644, PN 202 791, the Furoxan Hybrids [**24-38**] and Compounds **68a-c**)

An earlier study [Vo *et al.*, 1995] showed that the 2-pyridinyl compound (**68a**) acted only as a calcium channel antagonist, whereas the 3-pyridinyl (**68b**), 4-pyridinyl (**68c**) and BAY K 8644 racemates exhibited a calcium channel agonist effect on guinea pig ileum longitudinal smooth muscle (Table 4.3) (Figure 4.3.2.2.2b). Accordingly, replacement of the ester "methyl" group of BAY K 8644 by a [3-benzenesulfonyl)furoxan-4-yloxy]alkyl moiety (**33-35**) abolished the undesirable contractile agonist effect of BAY K 8644 on guinea pig ileum longitudinal smooth muscle. In contrast, similar replacement of the ester "isopropyl" group of the C-4 3-pyridinyl isomer (**68b**) to afford compounds **27-29**, or the 4-pyridinyl isomer (**68c**) to afford compounds **30-32**, did not abolish their contractile agonist response (EC_{50} = 1.44 to 15.08 μM) relative to BAY K 8644 (EC_{50} = 0.23 μM). In this latter assay, the contractile response (EC_{50} value obtained from a dose-response curve) induced by the test compound on guinea pig ileum longitudinal smooth muscle in the absence of carbachol is compared to the reference compound carbachol which is defined as producing a 100% increase in contractile force on GPILSM at a 0.167 μM concentration. The observation that the C-4 2-pyridinyl compound (**24**) induced an agonist effect on guinea pig longitudinal smooth muscle in the absence of carbachol (EC_{50} = 4.14 μM), relative to the corresponding isopropyl ester (**66a**), which does not induce an agonist effect, was unexpected. However, when the same assay was repeated using buffer containing 3.2 mM L-cysteine that enhances the release of nitric oxide (66% nitric oxide release in the presence of L-cysteine and 1% in the absence of L-cysteine), compound **24** retained its calcium channel antagonist effect on guinea pig ileum longitudinal smooth muscle (IC_{50} = 1.30 ± 0.34 μM, n = 3), but its undesirable agonist effect in the absence of carbachol was abolished due to increased release of the muscle relaxant nitric oxide. A similar experiment showed that the presence of L-cysteine also abolished the small agonist effect (12.7% increase in contractile force at 2.99 μM) induced by the C-4 4-benzofurazanyl compound (**35**) in the absence of carbachol, and that the calcium channel antagonist effect on guinea pig ileum longitudinal smooth muscle was increased[31]. It was concluded that nitric oxide diminishes (or abolishes) the undesired contractile effect on smooth muscle induced by

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[31] IC_{50} was > 11.96 μM (IC_{40} = 11.96 μM) in the absence of L-cysteine. IC_{50} was 8.26 ± 1.57 μM (n = 3) in the presence of L-cysteine.

1,4-dihydropyridine calcium channel agonists, and has a synergistic effect with 1,4-dihydropyridines on smooth muscle relaxation.

Previously, it was reported that the racemic C-4 2-pyridinyl (**68a**), 3-pyridinyl (**68b**), 4-pyridinyl (**68c**) compounds, and BAY K 8644, exhibited cardiac calcium channel agonist activity (positive inotropes) in an *in vitro* guinea pig left atrium assay [Vo *et al.*, 1995], where the calcium channel agonist activities were measured as the molar concentration producing 50% (EC_{50}) of the maximum contractile response produced by the test drug on guinea pig left atrium as determined from the dose response curves (Table 4.3) (Figure 4.3.2.2.2a). The *in vitro* calcium channel agonist activities (positive inotropic effect) for related compounds in which the ester "methyl" group of BAY K 8644, and the ester "isopropyl" group of PN 202 791, **68a**, **68b** or **68c**, was replaced by a [(3-benzenesulfonyl)furoxan-4-yl]alkyl moiety were determined. With the exception of the more potent compound **33** having a C-4 2-trifluoromethylphenyl substituent, compounds possessing a C-4 2-trifluoromethylphenyl (**34-35**), 4-benzofurazanyl (**36-38**), or 2-pyridinyl substituent (**24-26**) generally exhibited weaker cardiac positive inotropic activity (EC_{50} = 11.68 to > 44.66 μM) than related analogues having a C-4 3-pyridinyl (**27-29**) or 4-pyridinyl (**30-33**) substituent (EC_{50}'s predominately in the 3.02 to 8.84 μM range). These differences in positive inotropic activities could be due to a number of possibilities that include differences in the drug-receptor interaction, and/or preferential affinity for or access to the resting, open, or inactivated states of the L-type calcium channel receptor [Perez-Reyes & Schneider, 1994], the extent of nitric oxide released, and differential modulation by the two enantiomers for these racemic compounds [Shan & Knaus, 1999].

4.3.2.2.3 Piperazinyl, *N*-Nitrosopiperazinyl and N^1-Substituted Diazen-1-ium-1,2-diolate Hybrids

The piperazinyl and *N*-nitrosopiperazinyl 1,4-dihydropyridines (**43-44** and **46-48**) exhibit an overall equipotent calcium channel antagonist activity (IC_{50} = 0.22 to 1.16 μM range) that is weaker than the reference drug, nifedipine (IC_{50} = 0.01 μM) (Figure 4.3.2.2.3). The addition of a large diazen-1-ium-1,2-diolate group to a related piperazinyl moiety may have reduced the "fit" of the molecule in the receptor (**57**, IC_{50} = 1.19 μM). Moreover, the 4-pyridinyl *N*-nitrosopiperazinyl hybrid (**48**) shares a similar weak antagonist activity (IC_{50} = 1.16 μM) with compound **57**, because hybrid **48** has the largest molecular volume relative to the other piperazinyl and *N*-nitrosopiperazinyl

compounds (**43-44** and **46-47**). Indeed, the general order of calcium channel antagonist potency of the diazen-1-ium-1,2,-diolate's N^1-substitution suggests that a smaller molecular volume methyl substituent (**58-59**) (IC_{50} = 0.11 to 0.37 μM) is preferred for calcium channel antagonist activity relative to an ethyl (**60-62**) (IC_{50} = 0.82 to 3.35 μM) or *n*-butyl (**63-65**) substituent (IC_{50} = 0.27 to 0.85 μM).

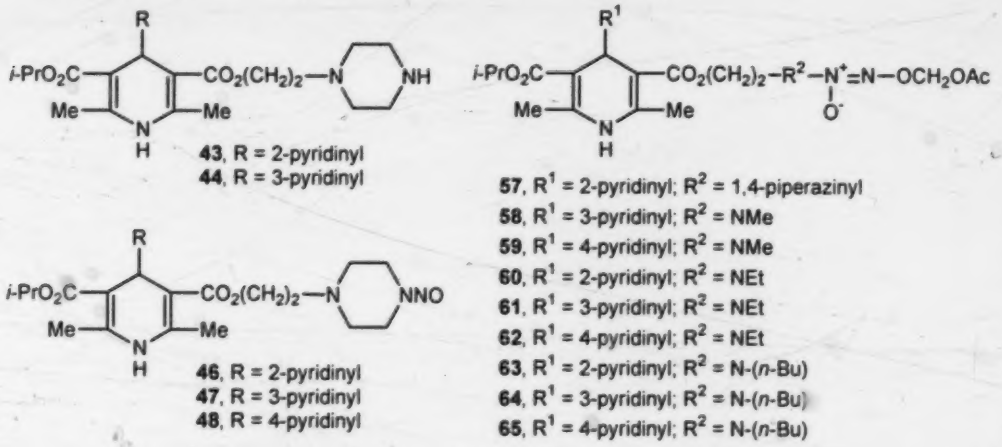


Figure 4.3.2.2.3: Piperazinyl, *N*-Nitrosopiperazinyl and N^1-Substituted Diazen-1-ium-1,2-diolate Hybrids of 1,4-Dihydropyridine Calcium Channel Antagonists (**43-44**, **46-48** and **57-65**)

It is not likely that there were sufficient concentration of esterases in guinea pig ileum longitudinal smooth muscle to hydrolyze and liberate nitric oxide during the calcium channel antagonist assay. Thus, the 1,4-dihydropyridine hybrids' molecular volumes remained intact throughout the *in vitro* assay.

A comparison of the effect which the point of attachment of isomeric C-4 pyridinyl substituents has on calcium channel antagonist activity shows the general relative activity order is 2-pyridinyl ≥ 3-pyridinyl > 4-pyridinyl, which concurs with the same observations made for the organic nitrate hybrids (**6-17**) (Section 4.3.2.2.1).

4.3.2.3 A Quantitative Structure-Calcium Channel Antagonist Activity Relationship Equation

Deriving a multiple collinear regression equation for calcium channel modulatory activity is more difficult than correlating with nitric oxide release. When it comes to 1,4-

dihydropyridine calcium channel modulators, structure-activity relationship analyses are difficult because of the problems of multicollinearity of substituent parameters, a high-leverage point, and position-dependent grouped observations [Mager *et al.*, 1992] (Section 1.4.1). When all the compounds synthesized in these studies are used to generate one equation, difficulties associated with a high-leverage point and position-dependent grouped observations are minimized. However, the multicollinearity of substituent parameters is still problematic. As discussed in Section 4.3.2.2.1, it was observed that the sizes of the C-3 and C-5 ester moieties provide interdependent steric contributions to the 1,4-dihydropyridine receptor binding. Traditionally, quantitative structure-activity relationship equations are derived from the physicochemical properties of a substituent. However, multicollinearity can be minimized if one derives "scores" for each substitution as described previously in deriving equations for nitric oxide release (Section 4.3.1). In fact, rather than using physicochemical properties to define the equation, scores that are derived from pharmacological data are used to form the regression equation.

The previous method of deriving multiple linear regression equations (Section 4.3.1.2) needs to be more robust, because multicollinearity is more apparent in calcium channel antagonism than in nitric oxide release. Although it is known that parameters may influence each other [Mager *et al.*, 1992], the interdependencies may be so small that they are within error ranges. The equation encompasses four parameters: C-3, C-4 and C-5 substituents, and nitric oxide release. The method for deriving scores for C-3, C-4 and C-5 substituents are identical.

In this regard, the scoring of the C-4 parameter will now be discussed, because the C-4 parameter has the most comparative data and is the most complicated. All calcium channel antagonist results are converted to –log (IC_{50}) values such that a high number denotes a more potent drug. The compounds are organized into groups of compounds that have the same C-3 and C-5 substituents but different C-4 substituents. As an example, one can pick a group that contains compounds **46-48** that have the following respective –log (IC_{50}) values of 6.54 (2-pyridinyl), 6.67 (3-pyridinyl) and 5.94 (4-pyridinyl). The range of –log (IC_{50}) in this group is $r = 6.67 - 5.94 = 0.73$. The maximum (r_{max}) of the ranges of all the groups indicates an estimated weight of the C-4 parameter in the regression equation. In the case of the C-4 parameter, r_{max} is going to be 1.34. The –log(IC_{50}) values are then scored as percentiles between 0 (the lowest value) and 1 (the highest value), thus, the values are 0.83 (2-pyridinyl), 1.00 (3-pyridinyl) and 0.00 (4-

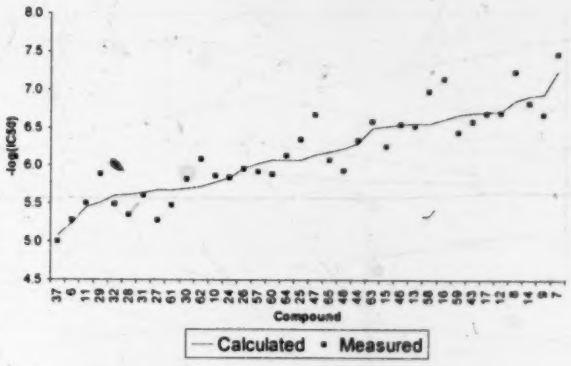
pyridinyl). A weight for this group is calculated by dividing r by r_{max} ($w = r \div r_{max} = 0.73 \div 1.34 = 0.55$). This weight ($w$) represents the reliability of the order of potency that is suggested by this group. The scores are weighed with w, such that the new scores are 0.45 (2-pyridinyl), 0.55 (3-pyridinyl) and 0.00 (4-pyridinyl). The group score for a 2-pyridinyl substituent is the average of the 2-pyridinyl scores in all of the groups. Three- and 4-pyridinyl substituents are scored in the same manner. The group scores are 0.47 (2-pyridinyl), 0.19 (3-pyridinyl) and 0.25 (4-pyridinyl). The group scores are weighed in relation to the regression equation with r_{max}, giving 0.63 (2-pyridinyl), 0.25 (3-pyridinyl) and 0.33 (4-pyridinyl). In the cases where there is insufficient comparative data to determine a score, the substituent is given the worst score (0.000).

As a mean of standardizing the extent of nitric oxide released, assigning a score for the "nitric oxide" parameter is done by ranking the extent of nitric oxide released relative to glyceryl trinitrate. Thus, the percentiles for compounds **6-16** are calculated by dividing the percent nitric oxide release by 11.43 (*N*-acetylcysteamine method), and the nitric oxide release data of the other compounds (**24-32**, **37**, **46-48** and **57-65**) are divided by 20 (L-cysteine method). The weight of the "nitric oxide" parameter is not calculated, in fact, it is a manually adjusted number such that the regression equation has the best linear fit.

Having done all the score assignments, a multiple collinear regression equation can be derived using Microsoft-Excel (2003) (Table 4.3.2.3). For aesthetic reasons, the constants k_n of the R^1, R^2, R^3, and "NO" parameters are combined with their respective scores (Figure 1.4.1b).

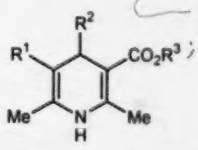
If the N^1-susbstituted diazen-1-ium-1,2-diolate hybrids (**57-65**) released nitric oxide during the calcium channel antagonist assay, the "NO" parameter will contribute to the regression equation. However, an accurate correlation equation cannot be derived when it is assumed that nitric oxide release occured. But an equation can be derived when it is assumed that nitric oxide is not released during the antagonist assays. This implies that negligible amount of nitric oxide was released during the calcium channel antagonist assays. Thus, it is plausible to speculate that there were insufficient esterases in the organ bath's physiological solution to liberate nitric oxide from the drugs.

Table 4.3.2.3: A Quantitative-Structure Activity Relationship Equation for Calcium Channel Antagonist Activity Exhibited by Compounds **6-16**, **24-32**, **37**, **46-48** and **57-65**



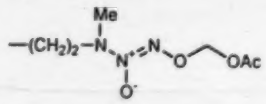
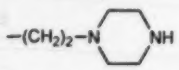
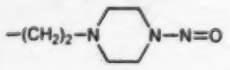
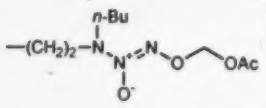
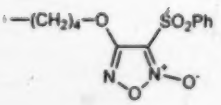
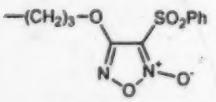
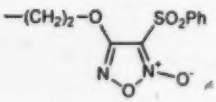
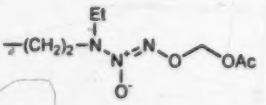
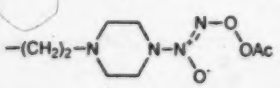
$$-\log(IC_{50}) = R^1 + R^2 + R^3 + 0.535\,(NO) + 3.278$$

$n = 36;\ R^2 = 0.85;\ p < 0.001$



R^1 (C-3 substituent)		R^2 (C-4 substituent)	
CO_2i-Pr	2.011	2-pyridinyl	0.663
CO_2i-Bu	0.542	4-pyridinyl	0.349
CO_2t-Bu	0.207	3-pyridinyl	0.263
CO_2Et	0.000	4-benzofurazanyl	0.000
NO_2	0.000		

NO = The extent of nitric oxide released relative to the percent nitric oxide released by glyceryl trinitrate

R^3 (C-5 substituent) (nitric oxide donor moiety)	
$-(CH_2)_2ONO_2$	1.300
	0.961
$-CH(CH_2ONO_2)_2$	0.933
$-(CH_2)_3ONO_2$	0.765
$-(CH_2)_4Cl$	0.757
	0.744
$-(CH_2)_4ONO_2$	0.599
	0.591
	0.472
	0.231
	0.220
	0.105
	0.048
	0.030

However, the equation has a better linear regression fit when it is assumed that the furoxan hybrids (**24-32** and **37**) released nitric oxide (R^2 improved from 0.60 to 0.85). This suggests that there were sufficient L-cysteine in the organ bath's physiological solution to liberate nitric oxide from the drugs. Although the amount of nitric oxide released was minute, an adequate amount of nitric oxide was released as to influence calcium channel antagonist activity.

In concurrence with the trend observed in the literature [Goldmann & Stoltefuss, 1991; Triggle, 1992], a $-CO_2i$-Pr substituent on the "essential side" C-3 or C-5 position provides optimal calcium channel antagonist activity. Indeed, it seems that a specific size improves activity such that the $-CO_2$Et moiety is too small, while the $-CO_2i$-Bu and $-CO_2t$-Bu moieties are too bulky to fit at the receptor site. Although, there were insufficient comparative data to assign a score to the nitro substituent, it is apparent that the IC_{50} for nitro-substituted compounds (**33-38**) are much higher than alkyl substituted compounds. In simpler words, 1,4-dihydropyridine calcium channel agonists have poor antagonist activities.

The regression equation suggests that the potency order with respect to the C-4 substituent is 2-pyridinyl > 3-pyridinyl and 4-pyridinyl. Moreover, the smaller pyridinyl substituents give rise to more potent calcium channel antagonists than the 4-benzofurazanyl substituted compounds. These patterns have also been observed in literature data for aryl groups (R^4) where an optimum antagonist activity profile is reported to be ortho > meta > para, and that activity is dependent of the size of the aryl group [Fossheim *et al.*, 1982; Bossert & Vater, 1989; Mager *et al.*, 1992; Triggle, 1992].

The multiple collinear regression equation indicates that although a size restriction is also present on the "non-essential side" of the 1,4-dihydropyridine, the requirements are not as stringent as that of the "essential side". The ranking for the nitrooxy compounds are $-(CH_2)_2ONO_2$ >> $-CH(CH_2ONO_2)_2$ > $-(CH_2)_3ONO_2$, $-(CH_2)_4Cl$ > $-(CH_2)_4ONO_2$, while the ranking for the piperazinyl compounds are piperazine > nitrosopiperazine >> 4-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)piperazine, and the ranking for the *N*-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-alkylamine is Me > *n*-Bu > Et. These patterns suggest that increasing the size of the substituents on the "non-essential side" of the 1,4-dihydropyridine ring decreases calcium channel antagonist activity. In contrast, altering the alkyl chain of the furoxan moieties does not influence calcium channel antagonist potency. One explanation for this irregularity is that the furoxan hybrids release nitric oxide while the other nitric oxide donor classes of

compounds do not. Indeed, the "NO" and R^3 parameters contribute 32% and 3%, respectively, to the regression equation in the cases of the furoxan hybrids[32] (**24-38**). Hence, the "NO" parameter is a stronger determinant of calcium channel antagonist activity than the R^3 parameter.

Meguro *et al.* (1985) reported that 1,4-dihydropyridine calcium channel antagonists having a piperazinylalkyl ester exhibited potent and long-lasting antihypertensive effects. The regression equation suggests that the incorporation of a diazen-1-ium-1,2-diolate moiety makes the drug too large, and drastically weakens calcium channel antagonist activity.

The range of each parameter in the regression equation indicates the general contributory order of each parameter. Indeed, the extent of nitric oxide released (2.0)[33] and "essential side" substitution (2.0) are the strongest determinants, followed by "non-essential side" (1.3) and C-4 heteroaryl substitution (0.7). Although C-4 heteroaryl substitution is the lowest determinant of calcium channel antagonist activity in the compounds investigated, a high-leverage point may have erroneously assigned such a poor rank since most of the compounds synthesized are mainly C-4 pyridinyl substituted. Moreover, the order of potency confirms that "non-essential side" substitution does not greatly affect calcium channel antagonist activity.

4.3.2.4 A Quantitative Structure-Smooth Muscle Agonist Activity Relationship Equation

An undesirable agonist effect of 1,4-dihydropyrinde calcium channel agonists is the non-selective contraction of smooth muscle. Muscular contraction in cardiac tissue induces a positive inotropic effect, while constriction of vascular smooth muscle promotes vasospastic hypertension. Ileal smooth muscle contraction can be measured by adding the test compound in the absence of carbachol to an organ bath containing a suspended guinea pig ileum longitudinal smooth muscle.

A multiple collinear regression equation was derived from the guinea pig ileum longitudinal smooth muscle agonistic data for the furoxan-calcium channel agonists **24** and **27-32** (Table 4.3.2.4). The simple method of assigning scores described in Section

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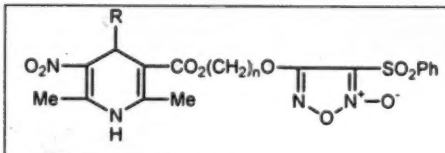
[32] Calculated as ([average R^3 scores] or [0.535 × average of "NO" values]) ÷ average of $-\log(IC_{50})$ for compounds **24-38**.

[33] Calculated as 0.535 × 3.80, where 3.80 is the "NO" value for compound **27**.

4.3.1.2 was used, because the sample size is small (n = 7). A correlation with the length of the alkyl-chain linking the 1,4-dihydropyridine and the furoxan moiety was observed. No correlation with the C-4 pyridinyl substituent, or the extent of nitric oxide released attributing to the undesirable smooth muscle contraction observed was evident. The equation may not be reliable because of the small sample size.

Table 4.3.2.4: A Quantitative-Structure Activity Relationship Equation for Undesirable Smooth Muscle Contraction Exhibited by Compounds **24** and **27-32**

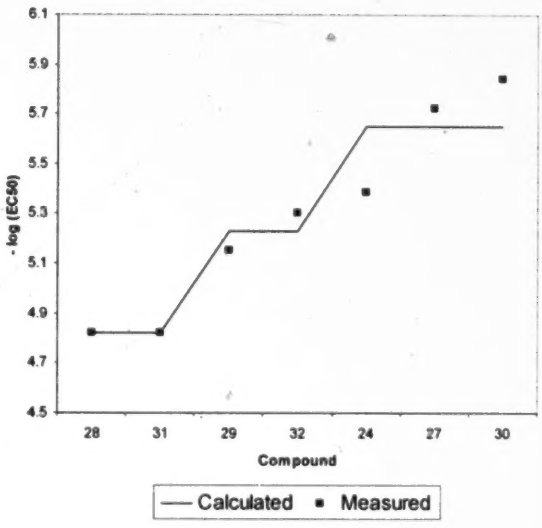


24, R = 2-pyridinyl; n = 2
27, R = 3-pyridinyl; n = 2
28, R = 3-pyridinyl; n = 3
29, R = 3-pyridinyl; n = 4
30, R = 4-pyridinyl; n = 2
31, R = 4-pyridinyl; n = 3
32, R = 4-pyridinyl; n = 4

$-\log (EC_{50}) = N + 4.822$

$n = 7$; $R^2 = 0.87$; $p = 0.002$

	N
n = 2	0.827
n = 4	0.407
n = 3	0.000



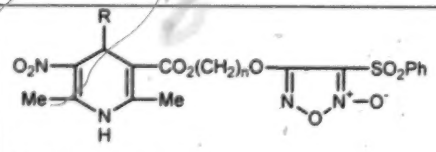
4.3.2.5 A Quantitative Structure-Positive Inotropic Activity Relationship Equation

A positive inotrope increases cardiac contractile force without increasing heart rate. Most 1,4-dihydropyridine calcium channel agonists are positive inotropes. All the calcium channel agonist-furoxan hybrids (**24-38**) prepared in this study display positive inotropic properties (EC_{50} = 3.02 to > 44.66 μM) that are weaker than the reference drug, BAY K 8644 (EC_{50} = 0.77 μM). More than a third of the compounds (**27**, **29-33**) synthesized are more potent than the reference drug, PN 202 791 (EC_{50} = 9.40 μM).

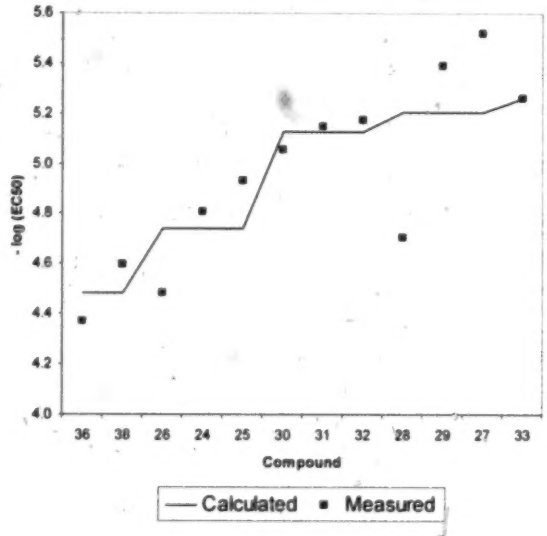
A multiple collinear regression equation was derived from the guinea pig left atrium calcium channel agonistic data for the furoxan calcium channel agonists **24-33**, **36** and **38** (Table 4.3.2.5). Once again, the simple method of assigning scores

described in Section 4.3.1.2 was employed, because of the small sample size (n = 12). There is a weak correlation between positive inotropy and C-4 (hetero)aryl substitution (R^2 = 0.65). No relationship of alkyl-chain length or the extent of nitric oxide release, contributing to the positive inotropic effect was evident. It is suspected that, much like the 1,4-dihydropyridine calcium channel antagonists, that interdependent effects of the C-4 and C-5 substituents contribute to positive inotropy.

Table 4.3.2.5: A Quantitative-Structure Activity Relationship Equation for Positive Inotropy Exhibited by Compounds **24-33**, **36** and **38**



24, R = 2-pyridinyl; n = 2
25, R = 2-pyridinyl; n = 3
26, R = 2-pyridinyl; n = 4
27, R = 3-pyridinyl; n = 2
28, R = 3-pyridinyl; n = 3
29, R = 3-pyridinyl; n = 4
30, R = 4-pyridinyl; n = 2
31, R = 4-pyridinyl; n = 3
32, R = 4-pyridinyl; n = 4
33, R = 2-trifluoromethylphenyl; n = 2
36, R = benzofurazan-4-yl; n = 2
38, R = benzofurazan-4-yl; n = 4



R	
4-benzofurazanyl	0.779
3-pyridinyl	0.723
4-pyridinyl	0.643
2-pyridinyl	0.259
2-trifluoromethylphenyl	0.000

$-\log (EC_{50}) = R + 4.482$

n = 12; R^2 = 0.65; p = 0.002

Calculating the regression equation using the complicated method of assigning scores as described in Section 4.3.2.3 will give at best a coefficient of determination (R^2) of 0.66 using three parameters; or 0.64 with two parameters; or 0.62 with one parameter (data not shown). Comparatively, the simple method of Section 4.3.1.2 derives a more valid equation (Table 4.3.2.5), because it provides a better linear regression fit (R^2 = 0.65) with the least number of parameters. The small sample size (n = 12) and the low coefficient of determination (R^2) suggest that more data is needed to improve the validity

of the equation. Removing compound **28** from the data set increases the coefficient of determination (R^2 = 0.89).

4.3.3 Computer Modelling and Dynamic Docking Experiments

Zhorov *et al.* (2001) and Zamponi *et al.* (2003) have each proposed a computer model of a calcium channel receptor protein (Section 1.4.1). The pseudo α_1-subunit models were derived from a potassium channel, KcsA. It was of interest to test the validity of the Zhorov and Zamponi models prior to docking selected 1,4-dihydropyridines (**6-17**, **24-38**, **43-44**, **46-48** and **57-65**) prepared in these studies at the modeled receptor site for the calcium channel.

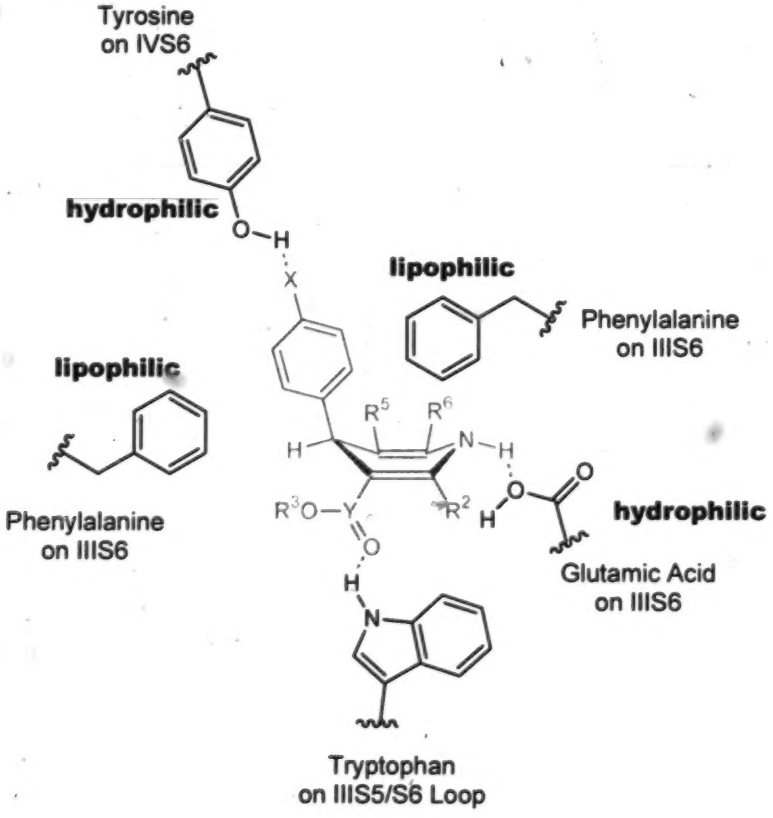


Figure 4.3.3a: The Zamponi Model – Proposed 1,4-Dihydropyridine Receptor Surface Binding Site

We reproduced the Zamponi's model on a core set (Insight II, Analysis, Biopolymer, Discover, Sketcher) of Accelrys programs running on a Silicon Graphics (SGI) R 14000 Octane 2 workstation. There was an inconsistency in Zamponi *et al.*'s work. Accordingly, we studied Zamponi *et al.*'s previous work [Natale *et al.*, 1999] and other literature sources from which their work was derived [Snutch *et al.*, 1991; Huber *et al.*, 2000], to clarify a contradictory assignment of a critical molecule-receptor interaction region that is described in their recent publication [Zamponi *et al.*, 2003]. Indeed, they reported that the C-4 heteroaryl substituent interacts with a tyrosine on **IV**S6 while displaying a diagram of the molecule interacting with a tyrosine on **III**S6. Their 1991 publication indicated that interaction occurs with the tyrosine on **III**S6 (Figure 4.3.3a). In their 2003 publication, Zamponi *et al.* docked 4-isoxazolyl 1,4-dihydropyridines in their receptor model. We reproduced their drugs and discovered that docking is favored (expressed in terms of van der Waals and electronegative interaction energies) when the isoxazolyl moiety is oriented toward the tyrosine on **IV**S6. We were skeptical of their results since these authors did not dock classical 1,4-dihydropyridines such as nifedipine or BAY K 8644 in their receptor model. We docked nifedipine and BAY K 8644 in their receptor model and discovered that neither of the drugs docked favorably, because there were too many plausible docking sites. We docked a larger calcium channel antagonist, amlodipine, and it docked favorably. We concluded that the Zamponi model was not adequately accurate for our research purposes.

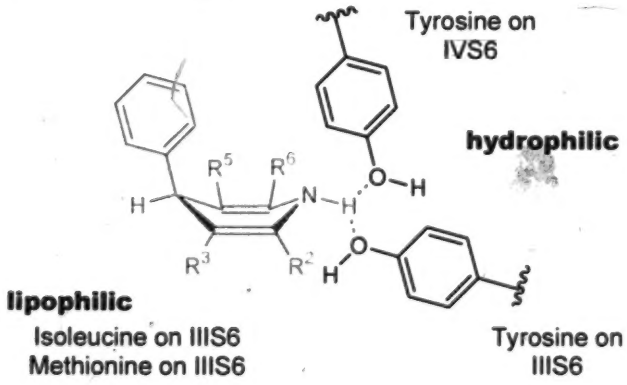


Figure 4.3.3b: The Zhorov Model – Proposed 1,4-Dihydropyridine Receptor Surface Binding Site

In their publication, Zhorov *et al.* (2001) docked nifedipine and both enantiomers of BAY K 8644 in their receptor model (Figure 4.3.3b). We reproduced their receptor model and were able to dock the (*R*)-enantiomers of compounds **6-17**. In general terms, the (*S*)-enantiomers are dominantly agonists while the (*R*)-enantiomers are dominantly antagonists [Mahmoudian & Richards, 1986a; Triggle & Rampe, 1989; Triggle, 1992]. We concluded that the Zhorov receptor model could accommodate larger molecules. In fact, nifedipine, BAY K 8644 and compounds **6-17** all shared the same docking position and orientation. Moreover, in agreement with Zhorov's reports, we observed that the 1,4-dihydropyridine NH protons of compounds **6-17** were all within hydrogen bonding distance (less than 4 Å) to the hydroxy group of a tyrosine on IVS6, that the pyridinyl nitrogen lone pairs of the 2- and 3-pyridinyl compounds (**6-7**, **10-14** and **22**) were also within hydrogen bonding distance to the same tyrosine on IVS6 (Figure 4.3.3b). We concluded that the Zhorov model was accurate, because different 1,4-dihydropyridine calcium channel modulators could dock at the same position and orientation. However, we were unable to correlate energy of stabilization with pharmacological activity. Moreover, we also found remnant amino acid side-chains from the original potassium channel that were within hydrogen bonding distance to the 1,4-dihydropyridine NH and the nitrooxy moieties. Although different 1,4-dihydropyridine calcium channel modulators could dock in the Zhorov receptor model at similar positions and orientations, the Zhorov model needs further refinements so that calcium channel modulatory activity could be reliably correlated with energy of stabilization and topological data.

5 CONCLUSIONS

Nitrosothiols, organic nitrates, furoxans and benzofuroxans, and N^1-substituted diazen-1-ium-1,2-diolates represent different types of nitric oxide donors that had been investigated as drug hybrids [Gasco *et al.*, 1996; Wang *et al.*, 2002]. Nitrosothiol-dihydropyridine hybrids were not considered to be suitable candidates for the design of calcium channel modulation-nitric oxide donor hybrid drugs, because nitrosothiols were reported to be highly unstable [Gasco *et al.*, 1996; Wang *et al.*, 2002].

A group of racemic 3-(nitrooxyalkyl or 4-chlorobutyl) 5-alkyl 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**6-17**), 5-[(3-benzenesulfonyl)furoxan-4-yloxy]alkyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl, benzofurazan-4-yl, 2-, 3- or 4-pyridinyl)-5-pyridinecarboxylates (**24-38**), 3-isopropyl 5-(2-piperazinylethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**43-45**), 3-isopropyl 5-(2-[4-nitrosopiperazinyl]ethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**46-48**) and 3-isopropyl 5-(2-[(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)(*N*,*N*-dialkylamino or 4-piperazin-1-yl)]ethyl) 1,4-dihydro-2,6-dimethyl-4-(pyridinyl)-3,5-pyridinedicarboxylates (**57-65**) were synthesized using modified Hantzsch reactions (Section 3.1). The N^1-substituted diazen-1-ium-1,2-diolate hybrids (**57-65**) were the most unstable and difficult to synthesize (Section 4.2.4.3), followed by the 1,4-dihydro-3-nitropyridine calcium channel agonists (**24-38**) (Section 4.2.1.2.1).

5.1 The Extent of Nitric Oxide Released

The extent of nitric oxide released was determined (Section 4.3.1.1) (Table 4.3). Because organic nitrates and furoxans require a thiol group to release nitric oxide [Ferioli, *et al.*, 1995; Wang *et al.*, 2002], the organic nitrate hybrids (**6-16**) were tested in the presence of the thiol *N*-acetylcysteamine, while the furoxan (**24-38**) and *N*-nitrosoamine hybrids (**46-48**) were tested in the presence of L-cysteine. L-Cysteine induced a greater release of nitric oxide than *N*-acetylcysteamine. It was found that the extent of nitric oxide released was directly proportional to the L-cysteine concentration, and that the rate of nitric oxide release was different for different compounds. The N^1-substituted diazen-1-ium-1,2-diolate hybrids (**57-65**) only released nitric oxide in the presence of esterases present in guinea pig serum. It was observed that higher DMSO concentrations (5% v/v) denatured esterases.

The extent of nitric oxide released from compounds **6-16**, **24-38** and **57-65** ranged from 33 times less than, to 7.2 times more than the reference drug, glyceryl trinitrate (11% mol/mol using *N*-acetylcysteamine; 20% mol/mol using L-cysteine). The extent of nitric oxide released *in vitro* was dependent upon the specific nitric oxide donor moiety (N^1-[4-piperazinyl, ethyl, *n*-butyl] diazen-1-ium-1,2-diolate > [3-benzenesulfonyl]furoxan-4-yl > N^1-methyldiazen-1-ium-1,2-diolate > nitrooxy) (Section 4.3.1.6). *N*-Nitrosoamine hybrids (**46-48**) did not release nitric oxide (Section 4.3.1.4).

It was shown that nitric oxide release seemed to diminish (or abolish) the smooth muscle contraction contractile side-effect of 1,4-dihydropyridine calcium channel agonists (Section 4.3.2.1), and enhance calcium channel antagonist properties (Section 4.3.2.3).

5.2 The Structure-Activity Correlations with Calcium Channel Antagonist Activity

All of the 1,4-dihydropyridine calcium channel antagonists investigated (**6-17**, **24-38**, **43-44**, **46-48**, and **57-65**) exhibited lower calcium channel antagonist activity (IC_{50} = 0.03 μM to > 29.91 μM) than the reference drug, nifedipine (IC_{50} = 0.01 μM).

The C-3 alkyl ester substituent on the 1,4-dihydropyridine ring was a primary determinant of calcium channel antagonism (CO_2*i*-Pr >> CO_2*i*-Bu > CO_2*t*-Bu > CO_2Et > NO_2). In concurrence with the trend observed in the literature [Goldmann & Stoltefuss, 1991; Triggle, 1992], a specific size improved activity such the CO_2Et moiety was too small, while the CO_2*i*-Bu and CO_2*t*-Bu moieties were too bulky to fit at the receptor side. Accordingly, 1,4-dihydro-3-nitropyridine calcium channel agonists (**24-38**) had weak antagonist activities.

The point of attachment of the isomeric C-4 (hetero)aryl substituent was a determinant of calcium channel antagonist activity providing the potency profile 2-pyridinyl > 3-pyridinyl and 4-pyridinyl > 4-benzofurazanyl > 2-trifluoromethylphenyl (Section 4.3.2.3). These patterns had also been observed by others for substituents on a phenyl ring where optimum antagonist activity was reported as ortho > meta > para, and that activity was dependent on the size of the aryl group [Fossheim *et al.*, 1982; Bossert & Vater, 1989; Mager *et al.*, 1992; Triggle, 1992].

Calcium channel antagonism with respect to the C-5 substituent was 2-nitrooxyethyl > 2-[*N*-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-methylamino]ethyl

and 1,3-dinitrooxy-2-propyl > 3-nitrooxypropyl, 4-chlorobutyl and 2-piperazinylethyl > 4-nitrooxybutyl and 2-[4-nitrosopiperazinyl]ethyl > 2-[*N*-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-(*n*-butyl)amino]ethyl > 4-[3-(benzenesulfonyl)furoxan-4-yloxy]butyl and 3-[3-(benzenesulfonyl)furoxan-4-yloxy]propyl > 2-[3-(benzenesulfonyl)furoxan-4-yloxy]ethyl, 2-[*N*-(O^2-acetoxymethyldiazen-1-ium-1,2-diolate)-*N*-ethylamino]ethyl and 2-[4-nitrosopiperazinyl]ethyl (Section 4.3.2.3). These patterns suggested that increasing the size of the substituents on the "non-essential side" of the 1,4-dihydropyridine ring decreased calcium channel antagonist activity, and that a high release of nitric oxide enhanced the calcium channel antagonism effect.

5.3 The Structure-Activity Correlations with Smooth Muscle Contraction

An undesirable agonist effect of 1,4-dihydropyrinde calcium channel agonists is the non-selective contraction of smooth muscle. In this regard, we discovered stronger correlations with the C-5 ester than C-4 (hetero)aryl substitution (Section 4.3.2.4). The general smooth muscle contraction potency profile of the $CO_2(CH_2)_n$([3-benzenesulfonyl]furoxan-4-yloxy) moiety was n = 2 > 4 > 3.

5.4 The Structure-Activity Correlations with Positive Inotropic Effect

None of the 1,4-dihydropyridines calcium channel agonists (**24-38**) exhibited cardiac calcium channel agonist activities (EC_{50} = 3.02 to > 44.66 μM) that were greater than the reference drug, BAY K 8644 (EC_{50} = 0.77 μM). Compounds **27** and **29-33** were more potent than the reference drug, PN 202 791 (EC_{50} = 9.40 μM). Although the correlations were weak, the order of positive inotropic potency for compounds **24-38**, with respect to substituents at the C-4 position, seemed to suggest that 4-benzofurazanyl > 3-pyridinyl > 4-pyridinyl > 2-pyridinyl > 2-trifluoromethylphenyl (Section 4.3.2.5). Substitution at the C-5 position did not affect agonist activity to any significant effect.

5.5 Future Prospects

Two computer models of the calcium channel receptor protein's α_1-subunit had been proposed by Zhorov *et al.* (2001) and Zamponi *et al.* (2003). These two models were reproduced and used to model some compounds prepared in these studies (Section 4.3.3). Our studies indicated the Zamponi model to be inaccurate. Although, the Zhorov model was more accurate, it lacked the precision needed to correlate energy of stabilization and topology with calcium channel modulatory activity. Over time, the scientific community will publish new calcium channel receptor models. We intend to test each new model's validity. Moreover, we may derive original models when more details regarding the receptor become available.

With a larger sample size (more compounds), all the derived multiple collinear regression equations will become more reliable. In particular, a clear pattern of positive inotropic potency may be observed for 1,4-dihydropyridine calcium channel agonists if more data are available. Thus, as a potential future project, data from our research group's previous studies can be used to re-enforce the regression equations.

We are no longer interested in the organic nitrate and furoxan hybrids, because the organic nitrate hybrids (**6-16**) release an inadequate amount of nitric oxide (Section 4.3.1.2), and the furoxan hybrids (**24-38**) are possibly toxic to normal cell lines [Moharram *et al.*, 2004] (Section 4.3.1.3).

However, we are still interested in N^1-substituted diazen-1-ium-1,2,-diolates. It would be of interest to attach an aminoalkoxy substituent to either the C-2 or C-6 position to mimic the slow onset and long lasting calcium channel antagonist, amlodipine [Romero *et al.*, 2003] (Figure 1.4.1.5b). Furthermore, the aminoalkoxy substituent could serve as a point of attachment for an N^1-substituted diazen-1-ium-1,2-diolate. In simpler words, that 1,4-dihydropyridine-(N^1-substituted diazen-1-ium-1,2-diolate) hybrid is a prodrug that, upon release of nitric oxide, may share a similar calcium channel antagonist profile with amlodipine.

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